THE UNIVERSITY
of EDINBURGH

To V, R_0 to V?

Jonathon A. Siva-Jothy

A thesis submitted for the degree of Doctor of Philosophy

The University of Edinburgh

2019

Declaration

The work within this thesis is my own, except when stated at the beginning of each chapter. I use “We” throughout chapters 2-5, as these chapters are in the process of submission or already published.

Jonathon A. Siva-Jothy

Abstract

Outbreaks of infectious disease can be caused by only a few highly infectious individuals. These individuals are produced by variation in traits affecting contact between infected and susceptible individuals, the likelihood that contact results in infection and the duration of infection. High-risk individuals are difficult to predict because traditional assessments of disease transmission, such as R_0, rely on population averages that conceal the variation that produces high transmission-risk phenotypes. Contact rate between infected and susceptible individuals, is primarily determined by behaviour whereas physiological immunity is the main determinant of the likelihood that contact causes infection and infection duration. I characterise variation in traits affecting the determinants of disease transmission and use this to predict individual variation in disease transmission, *V*. Using the fruit fly, *Drosophila melanogaster*, and its viral pathogen *Drosophila* C Virus, I have found pervasive and complex effects of genetic and sex-specific variation, mating, and infection on suites of behaviours, physiological traits and outcomes of infection. Many of my results point to an individual's disease transmission potential being determined by genetic background and sex. Males, for example, typically survive DCV infection longer than females, however the amount of virus they shed is also determined by their genetic background. To predict how this variation could affect disease transmission dynamics, I simulated outbreaks of DCV in theoretical populations. These populations exhibited genetic and sex-specific variation based on my experiments and significantly affected population-level outbreak dynamics. Differences in these dynamics highlight potentially high-risk transmission classes of individuals, defined by their genetic background and sex.

Lay Summary

Outbreaks of infectious disease can sometimes be caused by only a few highly infectious individuals. This presents a problem to preventing the spread of disease as these individuals are often difficult to identify before outbreaks occur. By understanding the traits that make these individuals transmit so much disease we can hope to prevent the outbreaks they cause. Individuals can be highly infectious because of variation in three key traits. Firstly, they may be highly social, meaning that when they become infected, they are capable of infecting more individuals. Alternatively, when infected, they may release larger quantities of disease-causing agents into the environment. Finally, they may stay infected for longer, which gives them more opportunities to spread disease. In this thesis, I use the fruit fly, *Drosophila melanogaster*, to measure how common sources of variation affect these three traits and use these measures to predict how individuals might differ in how much disease they spread.

My experiments show that sources of variation that are seen in many other organisms, such as genetics and sex, cause significant amounts of variation in the traits that affect disease transmission. I also show that key behavioural traits such as how flies avoid sources of infection in the environment and how they interact with other flies change when individuals become sick. I use this information to simulate disease outbreaks and find that the differences caused by sex and genetics change how disease spreads.

These findings suggest that an individual's genetics and sex affect its ability to transmit disease. This may help identify high risk individuals before outbreaks occur.

I dedicate this thesis to the best person I know, my mum.

Acknowledgements

I would like to start by thanking my supervisor, Pedro Vale, whose guidance and seemingly inexhaustible patience made this thesis possible. Over the past four years, Pedro's openness, humour and compassion have made him far more than a PhD supervisor to me, and I will miss him dearly.

I would also like to thank the Vale, Obbard, Regan and Walling Lab groups at large, but particularly Vanika Gupta and Katy Monteith. There have been many nights where Vanika's support and mentorship substituted for confidence and seeing her in India couldn't have been better. Katy's organisation, advice and craic made hours (and hours) of lab work fly by. Thanks as well to Lauren White and Meggan Craft whose collaboration made my fifth chapter possible. Working with them in Minnesota and Edinburgh has been an absolute highlight of my PhD.

To the friends who held it down for me over the past four years and never let me forget the absurdity of working on fruit flies. Max, Sam Moore, Sam Morris, Joe and Arran, I wish we could all live in the same town again, you're the best. Special thanks as well to the Moffat Wayers, Oscar and Jen, who never grew tired of hosting me or my carbonara.

I'd also never have gotten here without Will, Mike and Debbie, and I will always appreciate their honesty, love and support. I feel proud to call them my family.

I'd finally like to thank Mo. None of this would be worth it without you - MU.

Publications

The following publications have arisen from this thesis:

- Siva-Jothy, J. A., Monteith, K.M., Vale, P. F. (2018) Navigating infection risk during oviposition and foraging in a holometabolous insect. *Behavioral Ecology*. Vol. 29 (6) pp.1426 -1435

See Appendices 7.1.2 for this manuscript in its published format.

The following has been submitted for review and deposited on *bioRXiv*:

- Siva-Jothy, J. A., Vale, P. F. (2019) Viral infection causes sex-specific changes in fruit fly social aggregation behaviour. *bioRXiv*. 630913. doi: https://doi.org/10.1101/630913

I contributed to the following papers during my PhD, but these do not form part of my thesis:

- Gupta V, Vasanthakrishnan RB, Siva-Jothy J, Monteith KM, Brown SP, Vale PF. (2017) The route of infection determines *Wolbachia* antibacterial protection in *Drosophila*. *Proceedings of the Royal Society B: Biological Sciences*. 284: 20170809.

- Vale, P.F., Siva-Jothy, J., Morrill, A., Forbes M. (2018) The Influence of Parasites on Insect Behavior. In *Insect Behavior: from mechanisms to ecological and evolutionary consequences*. OUP Oxford. pp. 274-291

- Siva-Jothy, J. A.; Prakash, A.; Vasanthakrishnan, R. B.; Monteith, K. M.; and Vale, P. F (2018) Oral Bacterial Infection and Shedding in *Drosophila melanogaster*. *JoVE (Journal of Visualized Experiments)*, (135): e57676–e57676.

See Appendices 7.5 for these manuscripts in their published format.

Table of Contents

Chapter 1: General Introduction

1.1 Introduction

Individuals differ markedly in their propensity to transmit infection to others. Many outbreaks of disease are often caused and maintained by a minority of individuals that contribute disproportionate numbers of secondary cases of infection (Lloyd-Smith et al., 2005; VanderWaal and Ezenwa, 2016; Woolhouse et al., 1997). This pattern of heterogeneity in disease transmission is so frequently observed that it has become generalised to the '20/80 rule'. The 20/80 rule denotes that during an outbreak of infectious disease, 80% of secondary cases are caused by just 20% of the population (Woolhouse et al., 1997). In extreme cases, an even smaller minority, known as 'superspreaders', accounts for this transmission (Lloyd-Smith et al., 2005). Despite their ubiquity however, the underlying causes of variation in transmission are poorly understood and disease control strategies are ill-equipped to respond to high-risk individuals. Focusing disease control strategies on high-risk individuals would be more efficient and cost-effective than generalist approaches (Lloyd-Smith et al., 2005; Matthews et al., 2006). This has pushed understanding the determinants of heterogeneity in transmission to the forefront of disease ecology and epidemiology.

A commonly used tool for assessing the outbreak risk of a particular pathogen or parasite in a susceptible host population is the basic reproductive number, R_0 (Anderson and May, 1981). R_0 is defined as the average number of secondary cases caused by an infected individual in a susceptible host population (Anderson and May, 1981). When greater than 1, R_0 predicts a disease will spread. When less than 1, it is predicted to die out. The simplicity of R_0 has made it an invaluable tool to quickly assess a population's outbreak risk. The value of R_0 is calculated by deconstructing disease transmission into three interacting components: (1) the contact rate between infected and susceptible individuals, (2) the likelihood that

contact will result in infection, termed 'infectiousness' and (3) the duration of the infectious period (VanderWaal and Ezenwa, 2016).

A major shortcoming of R_0 is that by reporting the population's average transmission, it conceals high risk individuals (Li et al., 2011; Lloyd-Smith et al., 2005; Ridenhour et al., 2014). Individual heterogeneity in disease transmission means that pathogens are less likely to invade a population however, should key high-risk individuals become infected, outbreaks could be explosive (Lloyd-Smith et al., 2005). Many individual's transmission potentials were underestimated during the 2003 outbreaks of SARS across South East Asia, in part due to the delayed onset of symptoms. The failure to identify infected individuals led to several superspreaders evading control methods, and as a result, one of these individuals went on to infect 76 other people with SARS (Shen et al., 2004).

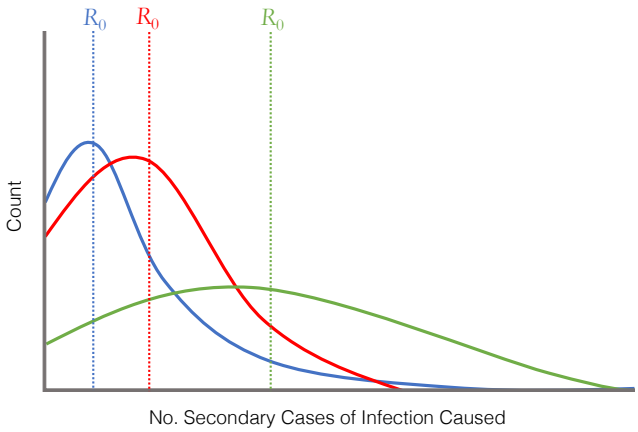


Figure 1. Distributions of the number of secondary cases of infection caused (V) by infected individuals in 3 theoretical populations distinguished by line colour. The population average of each population is marked by a dashed line and corresponds to that population's basic reproductive number (R_0), a traditional metric used to assess a population's outbreak risk. This figure serves to demonstrate the inability of R_0 to account for the dispersion of a population's distribution.

To tackle host heterogeneity in pathogen spread it is important to move beyond population averages and characterise individual variation in disease transmission. One approach is to apply the R_0 framework to individuals rather than populations. An individual's reproduction number, V, denotes the number of secondary cases produced by any individual in a given population (Lloyd-Smith et al., 2005). In a

population's distribution of *V*, the mean is equal to R_0 and high-risk individuals are present on the right-extreme tail (Figure 1).

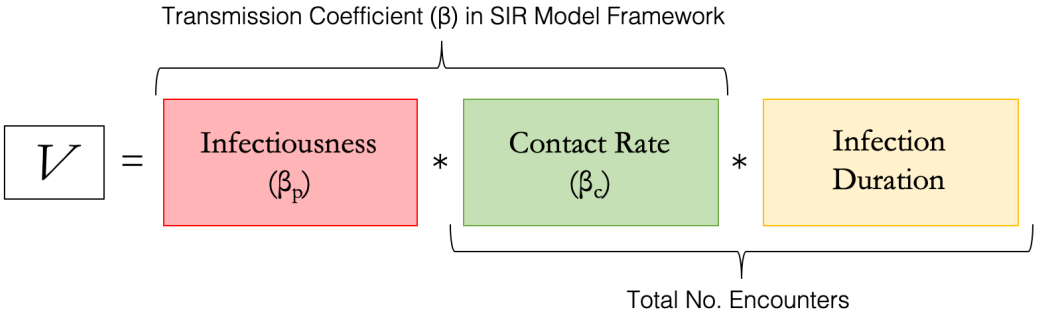


Figure 2. The components that underlie variation in the number of secondary cases of infection caused by an infected individual (*V*). Commonly used products of selections of these components have been annotated, such as β, which is used in SIR models to describe the rate infected individuals infect the susceptible population (Anderson and May, 1992). This figure is adapted from VanderWaal and Ezenwa (2016).

The sources of variation in *V*, can be broken down into traits affecting contact rate, infectiousness, and infection duration (VanderWaal and Ezenwa, 2016). Outside of host traits, environmental and pathogen factors can also affect determinants of *V*. High humidity, for example, alters the transmission efficiency of air-borne influenza by prolonging the virus' survival outside of a host (Loosli et al., 1943). The importance of environmental factors are evident in the seasonal changes of transmission, which is seen in polio, influenza and cholera (Altizer et al., 2006; Grassly and Fraser, 2006). Pathogen factors also affect transmission dynamics, strains of dengue virus affect genotypes of their mosquito vector differently, growing and establishing in salivary glands faster in certain genotypes (Fontaine et al., 2018; Lambrechts, 2011). Hosts are infected with a broad taxonomical range of pathogens and parasites. The environment this infection occurs in is also continuously changing. In the complexity of wild host-pathogen systems, the host population is the constant. In order to identify high-risk hosts, investigation must focus on the host factors that produce high levels of transmission. Although there are many examples of interplay between the two (Grassly and Fraser, 2006; Hawley et al., 2011), host behavioural traits primarily affect contact rate, and host physiological traits primarily determine infectiousness and infection duration (Grassly and Fraser, 2008;

VanderWaal and Ezenwa, 2016). Sex and genetics are two major sources of variation that determine the physiological and behavioural traits that define contact rate, infectiousness and infection duration. Moreover, an individual's sex and genes can be used as markers to identify high-risk individuals prior to outbreaks of infectious disease. In this chapter, I review what is currently known about heterogeneity in host behaviour and physiology in a broad range of host-pathogen systems. Each section primarily focuses on sex-specific and genetic variation, with broader sources of variation discussed as an endnote.

1.2 Behavioural Sources of Heterogeneity in Disease Transmission

Host behaviour primarily influences transmission by affecting contact between susceptible individuals and infected conspecifics or other sources of infection, e.g. pathogen-containing faeces (Grassly and Fraser, 2008; VanderWaal and Ezenwa, 2016). Regarding contact with infected conspecifics, these behaviours typically affect an individual's social group composition, social structure or mating system (Schradin, 2013).

1.2.1 Genetic variation in Contact Rate Behaviours

One of the earliest demonstrations of genetic variation in behaviour was found in the feeding behaviour of the nematode worm, *Caenorhabditis elegans*. In a relatively simple polymorphism, individuals tend to feed in groups or relative isolation. This feeding behaviour is determined by the *npr-1* gene, where specific isoforms are only present in socially and solitary-feeding individuals, respectively. The role of *npr-1*, in the determination of feeding behaviour is so strong that transgenic expression of the alternate variant changes the feeding phenotype expressed by the worm (de Bono and Bargmann, 1998). A similar polymorphism was identified in the nest building behaviour of the halictid bee, *Lasioglossum albipes*. Here, *syntaxin 1a* (a gene that mediates the release of synaptic vesicles) has been shown to affect whether the facultatively social bee builds solitary or eusocial nests (Kocher et al., 2018). *Syntaxin 1a* is highly conserved across the animal kingdom and has even

been implicated in human autism spectrum disorders (Shpigler et al., 2017). Similar homology is seen in expression of *vasopressin 1a receptor* (*avpr1a*). In the prairie vole, *Microtus ochrogaster*, *avpr1a* affects pair-bonding and patterns of monogamy versus polygamy (Hammock and Young, 2005; Winslow et al., 1993). *Avpr1a* appears to affect the same socio-behavioural traits in humans (Ebstein et al., 2010; Israel et al., 2008; Walum et al., 2008).

1.2.2 Sex variation in Contact Rate Behaviours

Sex-specific variation in social behaviours and contact networks are widely reported and have a number of consequences for the respective transmission risk of males and females. These differences have been shown to be sensitive to a number of factors. Social density, for example, has a major effect on the social interactions exhibited by male and female red deer (*Cervus elaphus*). Specifically, as population density increases, male social interactions become more transient, but occur more frequently. The transience of female social interactions on the other hand, does not change, and their frequency initially increases at intermediate densities before returning to their original levels at higher densities (Vander Waal et al., 2012).

Male-specific behaviours are often used to explain male-biased transmission. Many male mammal species for example, use scent cues in urine and faeces to assess social hierarchies. Should these excretions contain pathogens or parasites however, this behaviour increases transmission risk (Duneau and Ebert, 2012; Litvinova et al., 2010). Similarly, testosterone production has also been linked to male-bias in transmission due to its positive effect on contact rates. Artificially increasing testosterone production in male white-footed mice (*Peromyscus leucopus*) prolonged the maintenance of contact network connections (Grear et al., 2009). Similar effects of testosterone on sociality have also been seen in male red grouse (*Lagopus lagopus scoticus*), where male territory size increased with testosterone levels (Seivwright et al., 2005). Sex-specific behavioural changes post-infection have also been linked to male-biased transmission. When infected with *Mycoplasma gallisepticum*, males of the house finch, *Carpodacus mexicanus* show reduced

levels of aggression. As a result of this reduced aggression, uninfected males preferentially aggregate with infected individuals, while females show no such preference (Bouwman and Hawley, 2010).

While much rarer than male-bias, there are examples of variation in contact rate producing female-biased transmission in mammal host systems. When pregnant or lactating, female bank voles (*Mastophorus muris*), change their diet to accommodate the cost of gestation and parental care. A key change is an increase in the proportion of invertebrates in their diet. This increases female transmission risk as the habitats these invertebrates are found in contain more parasites and intermediate hosts (Grzybek et al., 2015). Female-bias is also seen in many invertebrate host species (Sheridan et al., 2000). Female-biased sexual transmission of the mite, *Parobia husbandi*, is also seen in the beetle, *Chrysophtharta agricola*. Here, males experience significantly more variation in mating success than females. As males that do not mate are not exposed to any transmission risk females are more likely to be infected (Seeman and Nahrung, 2004).

Many sex-specific behaviours are produced by sexual selection (Andersson and Simmons, 2006). Given these selection pressures often affect much more than behaviour, it is important to consider if the behavioural dimorphisms are accompanied by sexually dimorphic physiology. Sexual dimorphism in size, for example, is commonly observed in a range of species and can have a broad range of effects on transmission. For example. larger hosts are more likely to be infected by ectoparasites. This is seen in in the roe deer, *Capreolus capreolus*, where larger males also have greater tick burdens. Interestingly in the case of *C. capreolus*, sexual dimorphism in size accounts for the entire sex-bias in parasite prevalence (Kiffner et al., 2011). Another broad factor central to male and female differences in contact rate is the sex-ratio of a population. Sex-ratios can introduce another caveat to examples of sex-biased transmission. The transmission of tuberculosis in humans, for example, is characterised by male-biased prevalence, this may be less

relevant however in countries with male-skewed sex ratios such as China and India (Rhines, 2013).

1.2.3 The Effect of Infection on Contact Rate Behaviours

Contact rate can also be affected by the host response to environmental cues of pathogen presence even before infection occurs through infection avoidance behaviours. By preventing infection from taking place, avoidance behaviours circumvent the costs associated with both infection and mounting an immune response (Curtis, 2014). Should avoidance mechanisms fail, infection can elicit a number of changes to host behaviour. Infection-induced behavioural changes can be caused as a by-product of pathology, by the reallocation of host resources in aid of the immune response (sickness behaviours; Hawley et al., 2011) or by the pathogen or parasite promoting its transmission (parasite manipulation; Heil, 2016).

1.2.4 Before Infection

Infection avoidance behaviours are the first line of defence against pathogens and parasites, allowing individuals to circumvent the cost of fighting infection altogether (Curtis, 2014). The gregarious Caribbean spiny lobster, *Panulirus argus*, for example, has been shown to avoid infection by reducing social interactions with infected individuals (Behringer et al., 2006). If individuals are continually exposed to pathogens and parasites however, the efficiency of avoidance mechanisms may become irrelevant. In many social organisms, social ranking and role can be an important mediator of exposure risk, and as a result, the importance of infection avoidance behaviours. In meerkats (*Suricatta suricatta*), while social grooming reduces burdens of macroparasites, it also facilitates the transmission of *Mycobacterium bovis* (Drewe, 2009). With subordinate individuals taking part in more allogrooming than dominant members of the social group, they are thought of as key transmitters of *M. bovis* infection (Drewe et al., 2011). In many eusocial insect species, the activity of forager castes outside of the nest also makes them far more likely to encounter pathogens and parasites (Cremer et al., 2007; Schmid-Hempel and Schmid-Hempel, 1993). Many eusocial insect species mitigate the effect of this

increased infection risk, worker castes are often comprised of older individuals (Cartar, 1992; Moroń et al., 2008; Woyciechowski and Kozłowski, 1998). Interestingly, this also introduces an age-bias in transmission risk.

Infection avoidance is utilised in a variety of contexts outside of social interactions. Spatial avoidance, for example, occurs when hosts avoid areas where parasites are detected or likely to be found. In the presence of parasitoid wasps, the spreadwing damselfly, *Lestes sponsa*, preferentially lays eggs deeper below the surface of ponds to reduce egg parasitism despite the greater energetic cost incurred by deeper aquatic oviposition (Harabis et al., 2015).

Monarch butterfly (*Danus plexippus*) migration is also suggested to be a mechanism of infection avoidance as migrating individuals experience less parasitism (Satterfield et al., 2015). However, as parasitism reduces flight capability (Bradley and Altizer, 2005), it should be noted that migration's negative effect on parasitism may be a by-product of the behaviour rather than its selective driving force. Hosts also avoid time periods of parasite activity which is known as temporal avoidance. In the presence of the diurnal phorid fly, *Neodohrniphora curvinervis*, whole colonies of the leaf-cutter ant, *Atta cephalotes*, can shift their foraging activities from daytime, to night-time (Orr, 1992). A number of species also avoid trophic infection by not eating infectious food (Alma et al., 2010; Fouks et al., 2011). The scarcity of cannibalism, for example, is thought to be explained by it increasing pathogen and parasite transmission. In tiger salamanders (*Ambystoma tigrinum nebulosum*), cannibalism was shown to positively correlate with parasite burden (Pfennig et al., 1991). *C. elegans* has also been shown to avoid a broad range of bacterial pathogens during foraging (Meisel and Kim, 2014a; Schulenburg and Ewbank, 2007).

1.2.5 After Infection

Should mechanisms of infection avoidance fail, and hosts become infected, infection can induce further suites of behavioural changes. Infection-induced

behavioural changes present an important problem to predicting contact rates between infected and susceptible individuals as they can undermine measurements of susceptible hosts in the absence of infection. While there are examples of behavioural changes with neutral effects on host-pathogen dynamics, many of these changes have been shown to aid host immunity or promote pathogen or parasite transmission (Córdoba-Aguilar et al., 2018). Sickness behaviours, are changes that benefit the host (Hart, 1988), and parasite manipulation are those that benefit the pathogen or parasite (Poulin, 1995).

Sickness behaviours reallocate resources from other key host functions, such as foraging or mating, to bolster the immune response (Hart, 1988; Johnson, 2002). Changes in activity or social interactions, for example, have been used in animal husbandry for many years to diagnose sickness in livestock (Hart, 1988; Johnson, 2002; Weary et al., 2009). As visual symptoms of infection, these behavioural changes offer a potentially useful, and non-invasive, tool to assay infection status and transmission risk in members of a population. To test the effect of an infection-response while avoiding confounding pathogen effects, sickness behaviours are often measured by challenging hosts with lipo-polysaccharide (LPS) or heat-killed bacteria. LPS-challenged honey bees (*Apis mellifera*), for example, mount a strong immune response and exhibit reduced locomotor activity (Kazlauskas et al., 2016). Similar patterns of lethargy and reduced activity following LPS-challenge have also been shown in birds (Rotiroti et al., 1981) and amphibians (Llewellyn et al., 2011). While sickness behaviours improve host fitness by aiding immunity, they can also incur costs as reduced task function may lead to missed mating (Ashby and Boots, 2015; Kolluru et al., 2009; Pai and Yan, 2003) or feeding (Adamo et al., 2010; Kazlauskas et al., 2016; Sullivan et al., 2016) opportunities. Alongside these costs, some sickness behaviours have also been shown to increase parasite transmission. In finches, lethargy has been shown to facilitate disease spread when infected individuals aggregate at feeding sites (Bouwman and Hawley, 2010). Despite these costs, the abundance of shared sickness behaviours across a number of animal

taxa provide clear evidence of their overall adaptive role (Moore, 2013; Sullivan et al., 2016).

Parasite manipulation is also seen in many host-pathogen systems and can affect pre-existing host behaviours as well as produce entirely novel behaviours (Poulin, 1995). When *Cordyceps* fungus, for example, infects ants and a number of its other insect hosts (Shang et al., 2015), it increases climbing behaviour (Fredericksen et al., 2017), and induces novel biting behaviour. Climbing and biting aid transmission by anchoring sporulating individuals above susceptible conspecifics (de Bekker et al., 2015). Similarly, in a broad range of mammals, rabies infection has been shown to increase aggression and induce hydrophobia (Fleming, 1872; Rupprecht et al., 2002). Parasite manipulation is central to a number of multi-host systems where intermediate hosts are manipulated to avoid predators less in order to facilitate transmission through predation (Berdoy et al., 2000; Curtis, 2014; Hughes et al., 2012). Rats infected with *Toxoplasma gondii*, famously become attracted to the odour of cat urine (Berdoy et al., 2000).

Despite longstanding appreciation and acknowledgement, the interplay between behaviour and infection is very much an emerging field, with many important open questions. Given the potential for parasite manipulation of risk-taking behaviours to increase predation by non-host species, a longstanding question in disease ecology is how parasites control behaviour so tightly. Furthermore, with respect to both parasite manipulation and sickness behaviours, very few studies consider how host behaviour prior to infection influences the extent of behavioural changes following infection (Barron et al., 2015). With behavioural and physiological covariation thought to play a key role in producing outbreaks of infectious disease (White et al., 2018), characterising infection-induced behavioural changes is central to understanding disease transmission.

1.2.6 Caveats of Relying on Contact to Study Disease Transmission

A major barrier to using contact networks in epidemiology is that they do not necessarily predict an outbreak's transmission network. While genetic or sex-based predispositions to social interactions are linked to transmission many extraneous factors can prevent transmission along contact network connections. The disparity between contact and transmission networks has been demonstrated using *E. coli* transmission within and between cohorts of African Elephants. Habitat overlap with other groups of elephants was a better predictor of the *E. coli* strain individuals were infected with than their social group. This transmission network appears to be the result of the indirect nature of *E. coli* transmission. Bacteria-containing faeces is shed and transmitted to conspecifics through permanent swamps rather than social interactions (Chiyo et al., 2014). Differences between contact and transmission networks can also arise through heterogeneity in other components of disease transmission. For example, should individuals at key points in the network be particularly resistant to infection, they can serve as bottlenecks to the spread of disease. These individuals can serve as 'cutpoints' between social groups as their removal can isolate transmission between subpopulations (Nunn et al., 2015; Salathé and Jones, 2010; VanderWaal and Ezenwa, 2016).

1.3 Physiological Sources of Heterogeneity in Disease Transmission

Differences between individuals in their physiology, including their immune responses, primarily influence disease transmission by affecting how infectious hosts are, and how long they are infectious for (Grassly and Fraser, 2008; Vander Waal et al., 2012). While infectiousness and infection duration often interact, they have relatively distinct effects on disease transmission. Infectiousness typically influences transmission through the quantity or quality of shed pathogens or parasites, whereas infection duration determines how long infected individuals have to make contact with, and transmit the pathogen to, susceptible individuals. A host's

body condition and immune response play central roles in determining infectiousness and infection duration.

Immune responses can aid the host response to infection through a number of routes. Mechanisms of resistance address the cause of infection by reducing the pathogen or parasite load inside the host. Alternatively, tolerance mechanisms treat the consequences of infection, preventing the action of pathogen or parasite metabolites or repairing damage cause by immunopathology. Hosts are not exclusively resistant or tolerant, but rather utilise mechanisms from both strategies to varying degrees. The combination of resistance and tolerance mechanisms deployed by hosts defines their defensive capacity (Schneider and Ayres, 2008).

By responding to distinct aspects of host-pathogen interactions, resistance and tolerance have important consequences for heterogeneity in disease transmission. Tolerant hosts are thought to be more likely to bear a greater transmission risk than resistant hosts due to their strategy addressing the symptoms, rather than cause, of the infection. In doing so, tolerant hosts act as reservoirs, maintaining the pathogen or parasite population. Mouse superspreaders of *Salmonella* are caused by a tolerance mechanism that reduces the level of inflammatory myeloid cells in the intestine, which subsequently promotes bacterial shedding (Gopinath et al., 2014). The inherent risk of tolerant hosts however, assumes that resistant hosts efficiently reduce pathogen load. In cases where resistance is ineffective, unchecked pathogen load could produce sudden bursts of transmission from resistant hosts. Additionally, immune responses can depreciate host condition via immunopathology (Graham et al., 2005). The damage caused to the host by immunopathology reduces the number of barriers to pathogens and parasite proliferation and transmission. This is exploited in humans by the Cholera-causing bacterial pathogen, *Vibrio cholorae*, which aids its faecal shedding by disrupting the water balance of the small intestine (Gill, 1977).

Two useful proximate measures of infectiousness and infection duration are pathogen/parasite shedding and survival time/time to clearance, respectively. The nature of these measures varies according to the life-history traits of the host-pathogen system. In bacterial and viral systems, shedding is measured using colony forming and infectious units, while parasite eggs are used in many macroparasite worm systems (Wilson et al., 2002). When infection is lethal or cannot be cleared, host survival time can act as a useful measure of infection duration. As survival represents the most severe outcome of infection, how it changes with infectious dose is often used as a measure of tolerance (Gupta and Vale, 2017; Lefèvre et al., 2011b). Measuring infection duration when hosts are able to clear infection is much more difficult. In the absence of visual symptoms of infection, such as mange in wolves (Almberg et al., 2015), measuring infection duration in systems with clearance requires continuous observation and sampling of infected individuals (VanderWaal and Ezenwa, 2016).

1.3.1 Genetic Variation in Physiological Sources of Transmission Heterogeneity

Genetic variation in shedding, a key aspect of infectiousness has been demonstrated in a number of species. Genotypes of the ribwort plantain, *Plantago laneolata*, have been shown to vary in the number of spores shed following infection with the fungal pathogen, *Podosphaera plantaginis* (Susi et al., 2015b). Interestingly this genetic variation also interacted with coinfection, where multiple by multiple *P. plantaginis* strains produced the greatest number of spores (Susi et al., 2015b). Strains of the oat species, *Avena sativa*, have also been shown to differ in spore shedding following infection with the crown-rust disease-causing fungus, *Puccina coronata*. The Otana strain was found to be of particular interest, producing significantly more spores that also have a greater infectious potential than any other strain tested (Bruns et al., 2012). The majority of studies that measure shedding, focus on the quantity of infectious material shed, rather than its quality. The infectious quality of the bacterial pathogen, *Holospora undulata*, has been shown to vary according to the density of the host population (Magalon et al., 2010). Pathogen or parasite quality may therefore represent another, as yet unchecked,

source of transmission heterogeneity. Another important aspect of the relationship between shedding and infectiousness is how quickly individuals begin shedding after becoming infected. Genetic variation in shedding latency has been observed using the Ramshorn snail, *Biomphlamaria glabrata*, following *Schistosoma mansoni* infection. Alongside genetic variation in shedding latency, inbred lines were also found to differ in the number of eggs shed (Tavalire et al., 2016).

Genetic variation in lifespan following infection and survival rates has also been characterised in a number of host-pathogen systems. For example, following infection with the protozoan parasite, *Ophryocystis elektroscirrha*, genotypes of monarch butterfly (*Danaus plexippus*) have been shown to vary. Interestingly, this genetic variation in mortality was not affected by the infectious dose of the parasite, indicating no genetic variation in this measure of tolerance (Lefèvre et al., 2011b). Being of central importance to agriculture and selective breeding programmes, many domesticated and farmed animals have also been studied for genetic variation in survival following infection. These early studies have identified heritability in survival that is indicative of genetic variation. Mortality in the Atlantic Salmon, *Salmo Salar*, following infection with the furunculosis-causing bacteria, *Aeromonas salmonicida*, is highly heritable (Gjedrem et al., 1991). Although considerably smaller, there is also a heritable component of the mortality of cattle to *Mycobacterium avium* infection (Koets et al., 2000).

1.3.2 Sex-Specific Variation in Physiological Sources of Transmission Heterogeneity

In section 1.2.2, I described a number of behavioural differences between males and females that contribute to heterogeneity in disease transmission. In this section, I will discuss sex differences in physiology that could produce similar variation. Sex-differences in physiology are apparent in the transmission of a number of human STDs. Although the underlying causes are not understood, in HIV-1, infected males are more likely to transmit the virus to females than vice versa (van Lunzen and Altfeld, 2014). Conversely in gonorrhoea, men are more likely to contract infection

than women (Hooper et al., 1978) as a result of sex-specific inflammation responses in the genital tracts (Edwards and Apicella, 2004). Males have also been shown to play a key role in transmission dynamics in the yellow-necked mouse, *Apodemus flavicollis* (Ferrari et al., 2004). Anti-helminthic treatment targeting males significantly reduced female parasite prevalence, treatments targeting females, had no effect on male parasite prevalence (Ferrari et al., 2004). While behavioural factors may contribute to this male-biased transmission, physiological differences appear to exert a greater influence with males more likely to be sick, and shedding more parasite eggs than females (Ferrari et al., 2004). One often proposed explanation of male-biased transmission is the elevated production of testosterone, which is known to have a number of immunosuppressive effects (Folstad and Karter, 1992; Mills et al., 2010). While testosterone may be important to many systems, as there are also examples of female biased transmission it is not the sole determinant of male-bias. For example, a greater proportion of female voles (*Microtus gryalis*) are infected with *Heligmosomoides polygyrus*, and females also shed more eggs than males (Sanchez et al., 2011). Female-biased shedding is also seen in the crustacean host *Daphnia magna*, where females release more bacteria (*Pasteuria ramosa*) spores at death (Thompson et al., 2017).

Sex-differences in lifespan are also common to many species, and can be relatively extreme (Duneau and Ebert, 2012). The lifespan of many male eusocial insects, for example, is relatively ephemeral, in some cases lasting days, whereas females of the same species can live for years (Boomsma et al., 2004). Sex differences in lifespan chiefly affect heterogeneity in disease transmission by affecting the duration of an individual's infectious period. For example, female flowers of *Silene latifolia*, typically live longer than male flowers, as they are the site of fruit development. However, as pollinators often vector disease, this increased lifespan creates female-biased disease prevalence as male flowers simply drop from the plant following infection. In this way, male *S. latifolia* contribute to transmission without suffering from infection (Kaltz and Shykoff, 2001). Although thought of as an endosymbiont, interesting examples of sex-specific mortality come from Wolbachia infection in the

majority of its insect hosts (Brownstein et al., 2003; Charlat Sylvain et al., 2007; Sakamoto et al., 2007). Across many of these hosts systems, there is significant male-biased mortality which is thought to be caused by Wolbachia being transmitted through vertical, mother-offspring transmission (Bandi et al., 2001).

1.3.3 Other Sources of Variation in Physiological Sources of Transmission Heterogeneity

A multitude of factors outside of genetic and sex-specific variation can also affect patterns of shedding and lifespan following infection. Below I provide a brief overview of sources of variation that potentially have broad relevance to many host-pathogen systems. Central to characterising transmission heterogeneity in wild systems is a grounded understanding in the many factors that can produce physiological variation. In this thesis however, I primarily focus on genetic and sex-specific sources of variation.

The importance of within-individual variation to transmission heterogeneity is beginning to be understood (Chen et al., 2013; Susi et al., 2015b; VanderWaal and Ezenwa, 2016). The faecal shedding of many avian intestinal parasites, for example, peaks in the late-afternoon (Brawner III and Hill, 1999; Martinaud et al., 2009). This broad trend is thought to be a result of the environmental conditions of the late-afternoon favouring parasite viability (Martinaud et al., 2009). In blackbirds (*Turdus merula*) infected with the protozoan parasite *Isospora turdi*, the late-afternoon's cooler temperatures and lower levels of UV radiation was shown to decrease parasite oocyst mortality (Martinaud et al., 2009).

Another common source of variation is the ability of individuals to gather nutritional resources from their environment. While nutrition is essential to host condition, it can have negative and positive effects on transmission. For example, when fed on supplemented diets, domestic canaries (*Serinus canaria*) infected with *Plasmodium relictum* had lower parasite loads and experienced less virulence (Cornet et al., 2014). Conversely, better host nutrition increased pathogen shedding in *D. magna*,

where the higher-quality diet was exploited by the pathogen for growth (Vale et al., 2013).

Age and senescence also affect a broad range of species and exert an important influence over host condition and physiological sources of transmission heterogeneity. Across many host systems, younger individuals are generally more susceptible to disease than older hosts (Garbutt et al., 2014; Grenfell and Anderson, 1985; Hoye et al., 2012; Izhar et al., 2015). This is particularly true for host species with adaptive immunity, as younger individuals have had less time to garner a competent adaptive response to infection. In species with adaptive immunity, age may therefore be a particularly important source of heterogeneity as adaptive immunity is central to the clearance of infection (Tizard, 2009). By affecting this arm of the immune response, age can jointly influence traits affecting infectiousness and infection duration.

1.4 Using Drosophila to Study Heterogeneity in Transmission

To study and understand the underlying causes of heterogeneity in disease transmission, physiological and behavioural traits have to be considered together. The fruit fly, *Drosophila melanogaster*, has been central to studying biology since 1910, when it was used to demonstrate principles of heredity by Thomas Hunt Morgan (Morgan, 1910). Today, *D. melanogaster* is one of the most extensively characterised organisms in the animal kingdom, having been used as a model system in immunity (Apidianakis and Rahme, 2009; Govind, 2008; Kounatidis and Ligoxygakis, 2012; Mussabekova et al., 2017; Neyen et al., 2014), development (Jennings, 2011; Tolwinski, 2017), behaviour (Baier et al., 2002; Camiletti and Thompson, 2016; Sokolowski, 2001) and, most notably, genetics (Ayroles et al., 2009; Bier, 2005; Hales et al., 2015; Klämbt et al., 1991). The fruit fly has been used to uncover striking examples of deep homology with many *D. melanogaster* genes having homologues in vertebrate species, including humans (Medzhitov et al., 1997; Pandey and Nichols, 2011; Quiring et al., 1994). This extensive toolkit makes *D.*

melanogaster an ideal system to study genetic and sex-specific sources of heterogeneity in disease transmission. Additionally, a number of bacterial, viral and fungal pathogen species have been characterised in *D. melanogaster*, all of which provide information for where important physiological and behavioural sources of host variation in disease transmission might be found (Bier and Guichard, 2012; Buchon et al., 2014; Govind, 2008; Lemaitre and Hoffmann, 2007).

1.4.1 Using Fruit Flies to Understand Behavioural Sources of Heterogeneity in Disease Transmission

While *D. melanogaster* is traditionally thought of as a relatively solitary species, a number of recent studies have shed light on complex social behaviours that are central to fly fitness. *D. melanogaster* has been shown to take part in a number of social behaviours, ranging from relatively simple social digging (Louis and de Polavieja, 2017) to the more complicated transmission of social information (Battesti et al., 2012). Many of these behaviours, have important consequences for contact rate within and between groups of *D. melanogaster*, affecting social organisation, structure and mating. Below, I describe the findings of studies that have used *D. melanogaster* to study genetic and sex-specific variation in, as well as the effect of infection on, fruit fly behaviour.

1.4.2 Genetic Variation in fruit fly Contact Rate Behaviours

D. melanogaster has been used to characterise the genetic variation underlying a range of behavioural traits (Edwards et al., 2006; Lee et al., 2017; Philippe et al., 2016; Shorter et al., 2015). One of the more historic examples is the *foraging* (*for*) polymorphism, which encodes the rover and sitter phenotypes of larvae (Sokolowski, 1980). The *for* polymorphism is identified by the striking difference in locomotion: rovers tend to traverse large areas during foraging, while sitters cover a much smaller area (Sokolowski, 1980). Host dispersal plays a key role in the transmission of pathogens between social groups and determines where they are shed in the environment. In addition to affecting dispersal, *for* has a number of consequences for the genotypic composition of groups of *D. melanogaster*. Larger

social groups are more likely to be comprised of sitters as rovers are more likely to leave food resources once they become overcrowded or depleted (Philippe et al., 2016). *For* has also been shown to affect the locomotor activity of adult *D. melanogaster* with the rover allele associated with increased locomotion after feeding (Pereira and Sokolowski, 1993). Adult locomotor activity has also been widely used to assay the role of genes in the circadian clock (Klarsfeld et al., 2003) which has identified a number of mutations central to determining when individuals are active during the day (Konopka and Benzer, 1971; Strauss, 2002). Alongside these mutations, studies of circadian rhythm have also uncovered natural patterns of genetic variation. The length of the promoter region of the clock gene, *period*, for example, affects circadian period which is intimately tied to how individuals respond to seasonal changes in the day and night cycle. Strikingly, the distribution of promoter regions across geographically distinct populations of *D. melanogaster* matched differences in seasonality and day length along these lines of latitude (Sawyer et al., 1997).

Broad forms of genetic variation have also been observed in a number of parameters of *D. melanogaster* sociality. Monitoring contact networks has implicated olfaction as playing a key role in social interactions and contact rates, with mutations reducing and disrupting contact network connectivity (Schneider et al., 2012). Alongside these mutants, wildtype strains form distinct contact networks according to the genetic background of surrounding individuals (Schneider et al., 2012). Multiple genetic backgrounds also differ in the number of individuals they preferentially aggregate with at food sites (Saltz, 2011), how closely they aggregate with these individuals (Anderson et al., 2016), and how quickly they make this decision (Lihoreau et al., 2016a). The genetic variation that has been shown in an array of *D. melanogaster* social behaviours highlights their potential use to predicting high-risk individuals.

1.4.3 Sex-Specific Variation in Contact Rate Behaviours in Fruit Flies

Sexual competition in *D. melanogaster* produces a number of behavioural differences between males and females that affect contact rate. While both males and females often mate multiple times during their lifespan, how the sexes compete for access to mates either directly or through resources differs greatly. In *D. melanogaster* male-male aggression gains them access to mates (Baxter et al., 2015; Chen et al., 2002; Dierick, 2007) and has been linked to a number of genes (Edwards et al., 2006). It is also affected by many other factors such as winner-loser effects (Liu et al., 2011; Penn et al., 2010; Trannoy et al., 2015) and resource availability (Lim et al., 2014). Male-male aggression is potentially central to transmission dynamics as successful males have far more connections in contact networks with both males they have fought, and females they have access to. Alongside female aggression being less costly, the costs associated with male-male aggression suggest males likely present a greater transmission risk, at least as a result of behavioural differences. Males have also been shown to stimulate female aggregation and oviposition site-choice by depositing the pheromone 9-tricosene (Lin et al., 2015). Were 9-tricosene, or similar pheromones secreted by infected males this may further exacerbate a male-bias in transmission risk.

1.4.4 The Effect of Infection on *D. melanogaster* Contact Rate Behaviour in Fruit Flies

Although a range of sources of variation in *D. melanogaster* contact rate behaviours have been characterised, the effect of infection is relatively understudied. How infection alters behaviour is central to understanding and predicting transmission as individuals with high-risk behaviours may express low-risk behaviours in the absence of infection. *Drosophila* C virus (DCV) (Arnold et al., 2013) and Kalithea virus (Palmer et al., 2018), for example, have been shown to produce marked reductions in the movement of *D. melanogaster* following infection. In the case of DCV, these changes are also affected by sex and the presence of the *Wolbachia* endosymbiont, which has also been shown to increase lethargy in males (Vale and Jardine, 2015). In these instances, measuring the movement of susceptible

individuals would have resulted in overestimations of their locomotion during infection. DCV infection has also been shown to reduce the feeding rates of female flies following infection (Vale and Jardine, 2016a) which may affect faecal shedding of DCV as feeding can increase defecation rates (Wayland et al., 2014). Reduced motivation to feed following DCV infection could therefore also reduce DCV transmission.

In the interest of predicting the behaviour of infected individuals before outbreaks occur, it is important to note that there are examples of behaviours that are not affected by infection. Behavioural fever, for example, is a preference for warmer microclimates during infection to aid immunity through thermoregulation. Despite the survival benefit of behavioural fever, infection with DCV does not alter the preference of *D. melanogaster* for warmer refuges (Arnold et al., 2015). Behaviours that do not change following infection are hugely useful as individuals can be measured well in advance of outbreaks. Unfortunately, as many behaviours change following infection, the publication bias for 'positive' results may prevent behaviours that are unaffected by infection from entering wider circulation in the literature, despite their importance. Behaviours that are not affected by infection, are typically included as a secondary result (Arnold et al., 2013; Panteleev et al., 2007; Vale and Jardine, 2015).

1.4.5 Using Fruit Flies to Understand Physiological Sources of Heterogeneity in Disease Transmission

The genetic tractability of *D. melanogaster* has proven essential to dissecting the determinants of immune mechanisms and relating them to other species (Kounatidis and Ligoxygakis, 2012). To this day, *D. melanogaster* still serves as a fundamental tool to study immunity. In 2011, part of the Nobel Prize in Physiology or Medicine was awarded to work that identified *Toll*, a gene crucial to bacterial immunity in *Drosophila* and homologous to *Toll*-like receptors in mice (Lemaitre et al., 1996; Poltorak et al., 1998). Alongside the identification of many immune genes, studies

using *D. melanogaster* have revealed extensive variation in many other outcomes of infection relating to disease transmission.

1.4.6 Variation in Pathogen Shedding in Fruit Flies

In contrast to the many studies that measure parasite or pathogen shedding in mammalian hosts to study disease transmission (Ferrari et al., 2004; Gopinath et al., 2014; Matthews et al., 2006), relatively few *D. melanogaster* studies have measured pathogen or parasite shedding (Habayeb et al., 2009a; Siva-Jothy et al., 2018b; Unckless, 2011) and those that do use shedding as a descriptor of pathology. Specifically, titres of Nora virus (Habayeb et al., 2009a) and micrograph evidence of *Drosophila innubila* Nudivirus (DiNV) (Unckless, 2011) in fly faeces have been used to infer faecal-oral transmission. Studies so far have not considered more quantitative variation in pathogen shedding which is central to transmission heterogeneity. This is puzzling given that faecal-oral transmission is proposed as a natural route of infection for a number of pathogens (Bou Sleiman et al., 2015; Ferreira et al., 2014; Habayeb et al., 2009a; Palmer et al., 2018). The conspicuous absence of pathogen shedding from the *Drosophila* literature is likely a result of most research focussing on survival or the activation of immune genes and mechanisms (Apidianakis and Rahme, 2009; Govind, 2008; Kounatidis and Ligoxygakis, 2012; Mussabekova et al., 2017; Neyen et al., 2014).

1.4.7 Variation in Lifespan Following Infection in Fruit Flies

Survival rate and lifespan following infection are some of the most studied outcomes of infection in *D. melanogaster* (Kuo and Williams, 2014; Magwire et al., 2012; Taylor and Kimbrell, 2007) as mortality represents the most severe outcome of infection. A number of sources of variation have been shown to affect the survival of *D. melanogaster* following infection with a range of pathogens and parasites.

1.4.8 Genetic Variation in Lifespan Following Infection

Genetic variation in lifespan has been demonstrated in *D. melanogaster* using a number of pathogens. Early studies demonstrated genetic variation by making

comparisons between geographically isolated populations of *D. melanogaster*. Genetic variation in survival following infection with the fungal pathogen, *Beauveria bassinia*, for example, was demonstrated using comparisons between genotypes from African and non-African countries (Tinsley et al., 2006). More recently, detailed genome-wide association studies (GWAS) have been used to characterise and identify key immune genes and patterns of heritability in survival following infection. A GWAS revealed a relatively simple genetic basis to lifespan following systemic infection with *Drosophila* C virus. Coupled with functional validation by RNAi, this GWAS revealed that a polymorphism in the *pastrel* gene was responsible for 47% of the heritability in lifespan following DCV infection (Magwire et al., 2012). A similar GWAS of oral infection with the bacterial pathogen *Pseudomonas entomophila* also found a significant genetic basis to survival and identified multiple modulators of immunity (Bou Sleiman et al., 2015). These modulators appeared to mainly influence gut health and pathogen load by reducing reactive oxygen species (ROS) activity and the prevalence of an ROS-inducing agent, while upregulating stem cell activity and resistance mechanisms (Bou Sleiman et al., 2015). Genetic variation in survival has been shown to interact with other sources of heterogeneity such as diet. Genotypes fed on diets with reduced levels of yeast had significantly lower survival rates than those able to feed on yeast *ad libitum*, following infection with *P. entomophila* (Kutzer et al., 2018).

Given that many of the genes involved in the host response and survival following infection with viral, bacterial and fungal pathogen are often distinct, individuals likely present different transmission risks in outbreaks of different diseases. To this end, instances of correlated or co-occurring susceptibility to multiple pathogens are of particular interest to identifying high-risk individuals as they suggest certain individuals may bear a high transmission risk during outbreaks of different pathogens. In *D. melanogaster*, genetic variation in lifespan following infection with the fungal pathogen, *Metarhizium anisopilae* was shown to be correlated with lifespan following *P.* aeruginosa infection (Wang et al., 2017). While lifespan following infection may be correlated, differences in the correlations of shedding or

contact rate behaviours between these genetic backgrounds may lead to differences in transmission risk.

1.4.9 Sex-Specific Variation in Lifespan Following Infection

Male and female *D. melanogaster* have also been shown to frequently differ in lifespan following infection. The direction of this sex-difference appears to vary with a number of factors such as the pathogen or parasite, age and mating status. For example, male-biased mortality has been demonstrated following Kalithea virus infection (Palmer et al., 2018), while female-biased mortality is seen following *B. bassiana* infection (Taylor and Kimbrell, 2007). The female immune response to *B. bassiana* also senesces at a faster rate than the male immune response, resulting in the extent of this difference increasing as individuals grow older (Kubiak and Tinsley, 2017). Sexual reproduction introduces yet more variability to *D. melanogaster* survival following infection. The asymmetrical costs of reproduction can vary according to a number of ecological factors, such as familiarity with conspecifics (Hollis et al., 2015), female sexual attractiveness (Long et al., 2009) and number of mating rivals (Bretman et al., 2013). There are also more proximal causes of variation in lifespan that occur via sex such as the seminal fluid released by males during copulation which actively harms females, and increases their mortality following infection (Short et al., 2012). With respect to disease transmission in natural settings, the effect of mating on infection duration is likely prolific as virgin females are relatively rare.

1.4.10 Other Sources of Physiological Variation in Disease Transmission

A number of sources of variation have been identified in *D. melanogaster* that affect a range of other traits that potentially contribute to transmission heterogeneity. The pathogen or parasite load of individuals, for example, is a key marker of resistance and tolerance and is thought to be central to shedding rates (McCallum et al., 2017). Many studies have found extensive genetic and sex-specific variation in pathogen or parasite load (Duneau et al., 2017; Habayeb et al., 2009b; Lazzaro et al., 2006; Short et al., 2012; Vincent and Sharp, 2014) and linked it to other outcomes of

infection. For example, an individual's ability to control bacterial load during the early stages of infection is a key predictor of survival. Interestingly, the immune response initially mounted by an individual appears to be determined stochastically (Duneau et al., 2017). This stochastic variation may also explain why there is no correlation in the pathogen load between infections with different species of bacteria (Lazzaro et al., 2006). A number of other, non-stochastic factors have also been shown to affect pathogen load such as mating in females which results in immunosuppression that enables greater bacterial loads following infection with species of *Providencia* (Short and Lazzaro, 2010). The route of infection also plays a key determining role in infection outcome. During systemic infection, thoracic, rather than abdominal injection, has been shown to further reduce survival due to injury (Chambers et al., 2014). Infection route also determines many aspects of host-pathogen coevolution. The Wolbachia endosymbiont, for example, reduces mortality following oral but not systemic infection (Gupta et al., 2017c).

1.5 Thesis Aims

In this thesis, I use *D. melanogaster* and DCV as a host-pathogen system to test of common sources of behavioural and physiological variation in key traits, and outcomes of infection. I use these data to make a number of inferences and predictions about individual heterogeneity in disease transmission and how this might affect population-level transmission dynamics, with a particular focus on genetic and sex-specific variation. The chapters of this thesis cover sources of variation that affect multiple stages of infection, covering infection avoidance behaviours, how host physiology and behaviour changes during infection and how this variation could impact population-level disease dynamics.

In chapter 2, I use larval and adult *D. melanogaster* to test the effect of infection on foraging in larvae and oviposition-site choice in adults. Using two choice chamber setups I also manipulate the infection status of individuals from a single wildtype strain of *D. melanogaster* to test how DCV alters infection avoidance. Healthy

mothers show an oviposition preference for non-infectious sites that is affected by infection and changes over time. Conversely, when foraging, larvae show no evidence of infection avoidance.

Chapter 3 focuses on behavioural changes that occur following infection, and how these changes differ according to the genetic background and sex of the host. I measure social aggregation and locomotor activity in males and females from ten genetic backgrounds. Social aggregation and locomotor activity are putative determinants of contact rate, affecting how individuals move in their environment and interact with nearby conspecifics. I identify genetic variation in both traits, and a male-specific effect of infection in social aggregation.

In chapter 4 I use males and females from the same genetic backgrounds as chapter 3 to test genetic and sex-specific variation in a number of physiological outcomes of infection that influence infectiousness and infection duration. These measures include the viral load and virus shedding of individuals during the first three days of infection and lifespan following infection and viral load at death. I then go on to integrate virus shedding and lifespan data with chapter 2's social aggregation data to produce a framework that estimates individual DCV transmission potential. Finally, I characterise patterns of genetic and sex-specific variation in the values of transmission potential provided by this framework.

Chapter 5 considers how the genetic and sex-specific variation identified in chapter 4 would translate to population-level dynamics of disease transmission. In a collaborative effort, I simulate a number of populations comprised of individuals with traits previously measured in the lab and measure the transmission dynamics after randomly infecting a single individual. These *in silico* experiments suggest the genetic and sex-specific variation we see in transmission potential is capable of affecting population-level dynamics. Across genetic backgrounds, males generally pose a greater transmission risk than females.

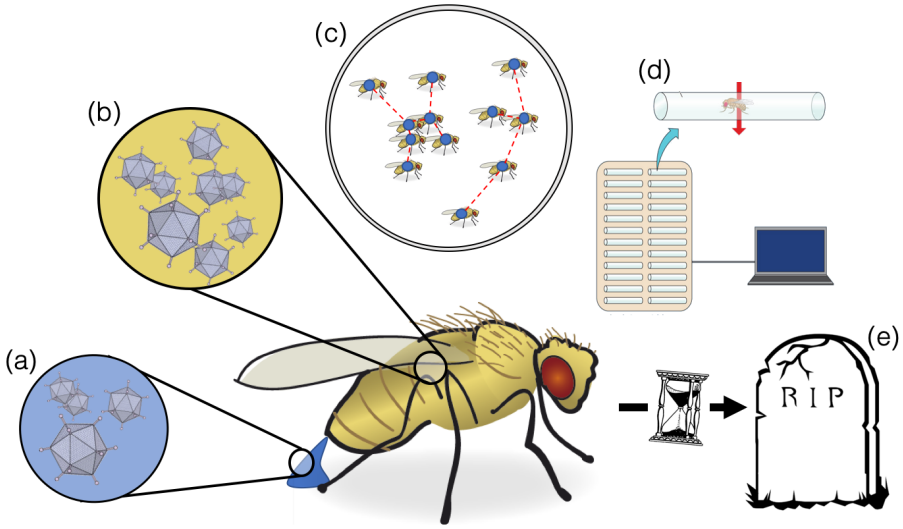


Figure 3. The suites of traits and infection outcomes measured in the experiments described in chapter 3 and 4. These were selected to represent the three core components of disease transmission and include (a) DCV shedding, (b) DCV load, (c) social aggregation, (d) locomotor activity and (e) lifespan following DCV infection. The estimations of individual transmission potential (V) that are described in chapter 4 were derived from measures of DCV shedding, social aggregation and lifespan following DCV infection.

Chapter 2: Navigating Infection Risk During Oviposition and Cannibalistic Foraging

The text of this chapter has been published (see Appendices 7.1.2).

Siva-Jothy, J. A., Monteith, K.M., Vale, P. F. (2018) Navigating infection risk during oviposition and foraging in a holometabolous insect. *Behavioral Ecology*. Vol. 29 (6) pp.1426 -1435

Katy Monteith conducted the larval foraging choice experiment described below and provided an initial draft of this experiment's materials and methods section. I am the sole author of the remaining text, with comments on earlier drafts from Pedro Vale and Katy Monteith.

Abstract

Deciding where to eat and raise offspring carries important fitness consequences for all animals, especially if foraging, feeding and reproduction increase pathogen exposure. In insects with complete metamorphosis, foraging mainly occurs during the larval stage, while oviposition decisions are made by adult females. Selection for infection avoidance behaviours may therefore be developmentally uncoupled. Using a combination of experimental infections and behavioural choice assays, we tested if *Drosophila melanogaster* fruit flies avoid infectious environments at distinct developmental stages. When given conspecific fly carcasses as a food source, larvae did not discriminate between carcasses that were clean or infected with the pathogenic *Drosophila* C Virus (DCV), even though cannibalism was a viable route of DCV transmission. When laying eggs, DCV-infected females did not discriminate between infectious and non-infectious carcasses and laying eggs near potentially infectious carcasses was always preferred to sites containing only fly food. Healthy mothers however, laid more eggs near a clean rather than an infectious carcass. Avoidance during oviposition changed over time: after an initial oviposition period, healthy mothers stopped avoiding infectious carcasses. We interpret this result as a possible trade-off between managing infection risk and maximizing reproduction. Our findings suggest infection avoidance contributes to how mothers provision their offspring and underline the need to consider infection avoidance behaviours at multiple life-stages.

2.1 Introduction

Behavioural immunity, the suite of behaviours that allow animals to avoid contact with infectious environments or conspecifics, is the first line of defence against infection (Curtis, 2014; Parker et al., 2011; Schaller and Park, 2011). Avoidance of infection relies on detecting cues of parasite presence - such as visual cues of infection risk or secondary pathogen metabolites - and integrating this sensory information to avoid sources of infection (Babin et al., 2014; Kacsoh et al., 2013; Kavaliers et al., 2004; Kiesecker et al., 1999; Kurz et al., 2017; Meisel et al., 2014; Stensmyr et al., 2012). In addition to external cues of infection risk, the internal state of the animal, including its physiological status as a result of prior pathogen exposure, may also affect the ability to detect and avoid infection (Curtis et al., 2011; Klemme and Karvonen, 2016; Vale and Jardine, 2016b).

Avoiding contact with pathogens allows healthy individuals to escape the pathology that results from infection, and also prevents the deployment of the immune response, which may be metabolically costly and even cause immunopathology (Curtis, 2014; Schaller and Park, 2011; Sears et al., 2011). Despite these clear advantages, avoiding infection completely is rarely possible. Foraging and feeding, for example, are vital aspects of host ecology, and are key to reproduction and fitness, but they are also major routes of pathogen transmission (Hall et al. 2007; Lefèvre et al. 2011).

Foraging and feeding are particularly important for holometabolous insect larvae, which devote most of their time to these behaviours. In situations of severe nutritional scarcity, larvae may even resort to cannibalism. For example, larvae of the fruit fly *Drosophila melanogaster* readily eat the carcasses of conspecifics following periods of starvation (Ahmad et al., 2015; Vijendravarma et al., 2013). Cannibalism may appear to be a beneficial strategy when the alternative is starvation but may increase the risk of trophic transmission of pathogens and parasites, especially if infected individuals are more likely to be targeted for cannibalism. While larvae of many

insect species are frequently observed to avoid infectious environments or food sources (de Roode and Lefèvre, 2012), it is currently unclear if trophic infection avoidance occurs during cannibalistic scavenging.

Beyond foraging during the larval stage, choosing where to oviposit or rear offspring is another important life-history decision, but can be risky if individuals are unable to identify and avoid potentially infectious environments. The environment in which adult insects choose to oviposit is therefore a major determinant both of offspring environmental quality and infection risk (Lefèvre et al. 2011; Lefèvre et al. 2012; Kacsoh et al. 2013). Infection avoidance by insects during oviposition has been observed in response to a number of parasites and appears to be driven by diverse sensory cues, including avoidance of parasitoid wasp visual cues (Kacsoh et al., 2013), and olfactory detection of bacteria and fungi (Kurz et al., 2017; Stensmyr et al., 2012). Together, both adult oviposition choice and larval food preference determine the likelihood of infection in the early life-stages of holometabolous insects, and therefore both behaviours play an important role in disease transmission dynamics (Ezenwa et al., 2016; Kiesecker et al., 1999).

Here, we investigate larval foraging and adult oviposition in a holometabolous insect - the fruit fly, *Drosophila melanogaster* - in the context of infection avoidance. Our study consisted of choice assays performed on either larval or adult stage *D. melanogaster*. Fly larvae were presented with a choice of scavenging on either a clean, non-infectious adult fly carcass, or a carcass that had been previously inoculated with a systemic *Drosophila* C Virus (DCV) infection (Figure 1a). In a second experiment, we tested adult oviposition choice by giving female flies the choice to lay eggs on a clean food source, a clean food source also containing a clean carcass, and a food source containing a carcass with a systemic DCV infection (Figure 1b). This 3-way choice assay allowed us to examine an important conflict faced by mothers: a carcass may present an additional nutritional source for future offspring but may also present a potential risk of infection. In both experiments

we assessed the fitness consequences of choices at both life-stages by following the development of larvae or laid eggs.

2.2 Materials & Methods

Fly Lines and Rearing Conditions

In both experiments we used laboratory stocks of *D. melanogaster* Oregon R (OreR). We kept fly stocks in plastic bottles (6oz; Genesee Scientific, San Diego, California, US) on a standard diet of Lewis medium (Lewis, 2014) at 18±1˚C with a 12 hour light:dark cycle. Stocks were tipped approximately every 21 days into new bottles. Before the experiments, we transferred flies to clean bottles and maintained them at low density (~50 flies per bottle) for a minimum of two generations at 25±1˚C with a 12-hour light:dark cycle.

Virus culture and infection

Drosophila C Virus (DCV) is a horizontally transmitted positive-sense ssRNA virus of the Dicistroviridae family (Huszar and Imler, 2008). DCV infection establishes in the digestive, reproductive and fat tissues, resulting in a range of behavioural and physiological pathologies in both larval and adult stage flies, including reduced locomotor activity, metabolic and reproductive dysfunction, and eventually death (Arnold et al., 2013; Chtarbanova et al., 2014; Gupta et al., 2017; Stevanovic and Johnson, 2015; Vale and Jardine, 2015). The DCV isolate used in this experiment was originally isolated in Charolles, France (Jousset et al., 1977) and was grown in Schneider *Drosophila* Line 2 (DL2) as previously described (Vale and Jardine, 2015b), serially diluted ten-fold in TRIS-HCl solution (pH=7.3), aliquoted and frozen at -80˚C until required. To infect flies, we bent Austerlitz insect pins (0.15mm in diameter) at a 90° angle ~0.5mm from the tip, dipped the tip in DCV, and inserted it into the soft tissue under the fly's wing, with the fly under CO_2 anaesthesia. Control infections employed the same protocol but with a needle tip dipped in sterile TRIS solution.

Infection avoidance during larval foraging

We had previously observed that fly larva would readily cannibalize dead adult fly carcasses (Video S1), and we hypothesized that cannibalism could be viable route of transmission. We would therefore expect selection for the avoidance of potentially infected carcasses, and so we tested if fly larvae could discriminate between healthy and potentially infectious fly carcasses. To generate these carcasses, we randomly selected 4-7-day old male and female flies from an age-matched population. For each sex, we stabbed half of the flies with DCV 10^7 DCV Infectious Units (IU)/ml and the other half stabbed with sterile TRIS buffer. Following 6 days (to allow viral replication), we froze live flies at -80 ˚C until required. We confirmed the infection status of the carcasses using DCV-specific qRT-PCR (see below) by randomly picking 5 male and 5 female flies.

We carried out a two-choice assay by placing ~100 fly eggs at the centre of each Petri dish containing ~20ml solid agar (5% sugar) and allowed the resulting 3rd instar larvae to forage towards either a clean fly carcass or a carcass infected with DCV, placed at an equidistant position from the eggs (3cm) (Figure 1a). We set up 56 'choice' assays where larvae could choose between a clean or DCV infected carcass, and 20 'control' assays, where both carcasses were clean (half of assays contained male carcasses, and the other half contained female carcasses). To differentiate between any effects of carcass degradation from a direct effect of DCV presence on larval choice, we also set up an additional 30 plates without fly carcasses, containing 10µl of DCV (10^7DCV IU/ml) and 10µl of TRIS (two-choice; N=20) or only TRIS (control; N=10). 18 of the 106 plates set up across all treatments were excluded from the final dataset due to damage to the surface of the agar which could have affected larval movement. We conducted all assays at 25±1˚C with a 12-hour light:dark cycle before being photographed after 72 hours. We marked images using Adobe Photoshop CS3 to count the number of larvae within each plate half and within an area immediately surrounding the carcasses/droplets (~2.2cm in diameter – see Figure 1a).

Larval infection status and virus quantification

We randomly selected 10 wandering-stage larvae found immediately adjacent to each carcass in 20 'choice plates' and one carcass in 6 'control plates' to assess DCV infection status and quantify viral load. We performed viral quantification using absolute quantification of DCV RNA copies using qRT-PCR. Total RNA was extracted by homogenizing the flies or larvae in TRI Reagent (Invitrogen, Carlsbad, California, US) and using Direct-zol RNA miniprep kit (Zymo Research, Irvine, California, US), including a DNase step. The eluted RNA was then reverse-transcribed with M-MLV reverse transcriptase (Promega, Madison, Wisconsin, US) and random hexamer primers, and then diluted 1:1 with nuclease free water. The qRT-PCR was performed on an Applied Biosystems StepOnePlus system using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, California, US) using the following forward and reverse primers, which include 5'-AT rich flaps to improve fluorescence (Afonina et al., 2007) (DCV_Forward: 5' AATAAATCATAAGCCACTGTGATTGATACAACAGAC 3'; DCV_Reverse: 5' AATAAATCATAAGAAGCACGATACTTCTTCCAAACC 3'; with the following PCR cycle: 95°C for 2min followed by 40 cycles of: 95°C for 10 sec followed by 60°C for 30 sec. Two qRT-PCR reactions (technical replicates) were carried out per sample. For absolute quantification of DCV, the concentrations of DCV in the samples were extrapolated from a standard curve created from a 10-fold serial dilution (1-10^{-6}) of DCV cDNA.

Larval development and infection status

To analyse the effect of foraging choice on larval development, we removed 15 larvae found within 2cm of infected carcasses from 20 'choice' plates and from uninfected carcasses on 6 'control' plates. We transferred larvae from each carcass together into plastic vials containing Lewis medium and recorded the number of larvae that developed into pupae and the number of eclosed adults. We froze a subset of these adults in TRI reagent and tested their infection status to verify DCV infection's persistence through metamorphosis

Infection avoidance during oviposition

Following our test of infection avoidance at the larval stage, we carried out a second experiment to test the oviposition preference of female *D. melanogaster* when presented with a choice of clean and potentially infectious oviposition sites. We made choice chambers by joining two bases of transparent plastic Petri dishes with adhesive tape, making a chamber 10cm in diameter and 2 cm in height. Chambers contained three oviposition sites comprised of upturned caps filled with Lewis medium, arranged in a triangle, each site, 50mm from the other two (Figure 1b). Oviposition sites contained either only Lewis medium, Lewis medium and an uninfected fly carcass, or Lewis medium and a DCV-infected fly carcass (infection protocol described above).

Three-day-old male and female flies were isolated as virgins and females were stabbed with either a virus-contaminated (10^8 infectious units (IU) per ml) or sterile, virus-free control solution. Following infection, females to be used in the oviposition assay were introduced to two uninfected males for mating for 72 hours. After which a single mated female was introduced to an oviposition chamber and placed at 25°C (12-hour light:dark cycle) to await oviposition. Two females (1 infected and 1 uninfected) laid no eggs during the experiment so were excluded from the final dataset. In total, we measured the oviposition choice of 80 females. As DCV has been reported to affect *D. melanogaster* fecundity (Thomas-Orillard 1984; Gomariz-Zilber and Thomas-Orillard 1993; Gupta et al. 2017), to account for differences in the total number of eggs laid by our infection treatment group we measured oviposition site choice by counting the number of eggs at each site rather than the proportion of eggs laid at the three respective sites. To count the number of eggs laid on each oviposition site, we took photos of individual oviposition sites with a Leica MC170 HD camera attachment on a Leica 0.32x/WD 200mm S8APO microscope (Leica microsystems, Wetzlar, Germany) after females had been in the chambers for 24 and 48 hours.

Fitness consequences of oviposition site choice

We quantified the potential fitness consequences of oviposition preference by transferring all oviposition sites, including carcass (if present), to individual vials and recorded egg-to-adult viability. We froze adults that eclosed from clutches during this experiment alongside one another in TRI reagent and DCV infection analysed using the same protocol as above. A total of 24 clutches were analysed in this way, we excluded 6 of these due to degradation or contamination during qPCR preparation.

Statistical Analyses

In the larval choice experiment, we analysed the proportion of larvae choosing a given plate half or carcass area; larval DCV titres; the proportion of larvae developing into pupae (logit transformed); and the proportion of pupae that developed into adult flies (logit transformed) and adult DCV titres. All response variables were analysed using Generalized Linear Models (GLMs) with 'carcass sex' and 'carcass infection status' and their interactions as fixed effects. In the adult oviposition experiment, we used the number of eggs laid at each oviposition site to assess infection avoidance. We analysed egg counts, rather than the proportion of eggs laid on each oviposition site, to account for potential differences in fecundity between infected and uninfected flies (Gomariz-Zilber and Thomas-Orillard, 1993; Gupta et al., 2017; Thomas-Orillard, 1984). The number of eggs laid was analysed using a generalized linear mixed model (GLMM) with Poisson distributed error. Our model used a full factorial 3-way interaction between oviposition site, maternal infection status and the 24-hour period eggs were laid. The total number of eggs laid, and the choice chamber were included as random effects, with the latter nested within the fly's infection status, to account for repeated measures. The proportion of eggs that later eclosed as adults (egg-to-adult viability) was analysed using a GLMM with a binomially distributed error, with oviposition site included as a fixed effect. All statistical analyses and graphics were carried out and produced in *R* 3.3.0 using the *ggplot2*, *lme4* and *multcomp* packages.

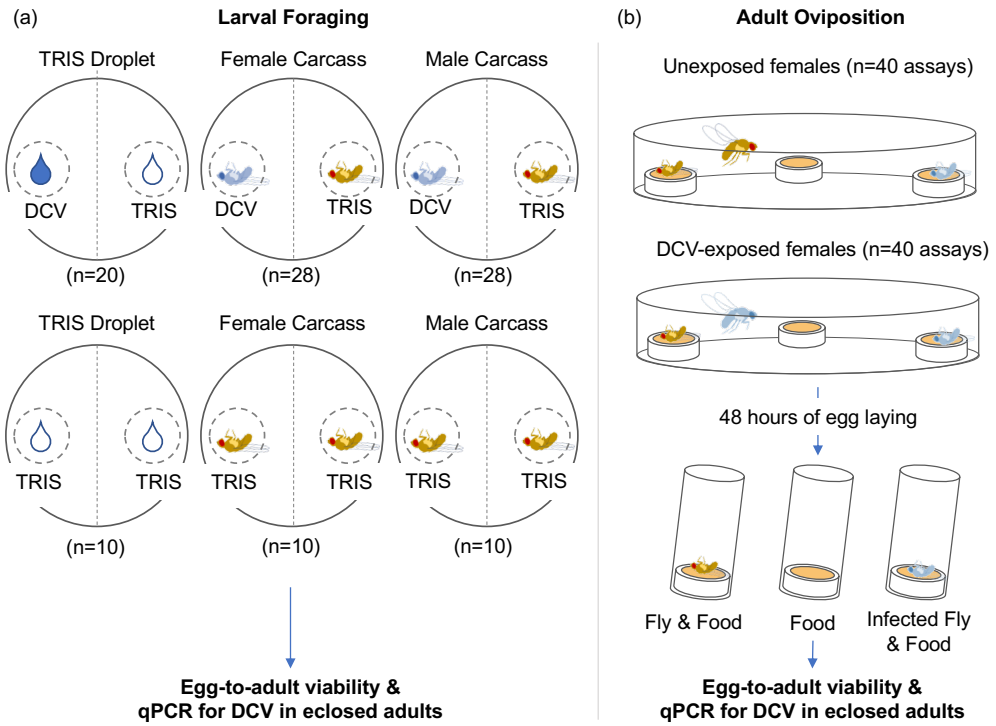


Figure 1 - Experimental design. (a) Two-choice chamber used to measure larval foraging choice when presented with infectious and non-infectious food sources and the life-history data collected after the 72-hour assay. Petri dishes were set up as either two-choice plates (containing an infectious and non-infectious food source) or control plates (containing only non-infectious food sources). Eggs were placed at the centre of each plate, allowed to hatch and left for 72 hours whereupon the position of larvae was recorded to assay infection avoidance. (b) Three-choice chamber used to assay oviposition site choice in infected and uninfected mothers when presented with three sites containing just food, food and a fly carcass and food and an infected fly carcass. The number of eggs laid at each site was measured twice at two 24-hour intervals. After 48 hours, oviposition sites were removed, and clutches were allowed to develop to adults whereupon the viral load of a randomly selected sub-sample was assayed.

2.3 Results

Larval flies do not avoid infectious food sources when scavenging

Fly larvae that hatched from eggs placed in the centre of the Petri dish, dispersed towards and consumed the fly carcasses placed at the edges of the dish. We found no evidence that fly larvae can avoid infected food sources. Regardless of the measure of preference (plate half larvae were found in or the area surrounding each carcass or TRIS droplet) larvae showed no significant preference for clean or infected fly carcasses (Figures 2a, 2b; Table 1 – Figure S1; Appendices 7.1.1).

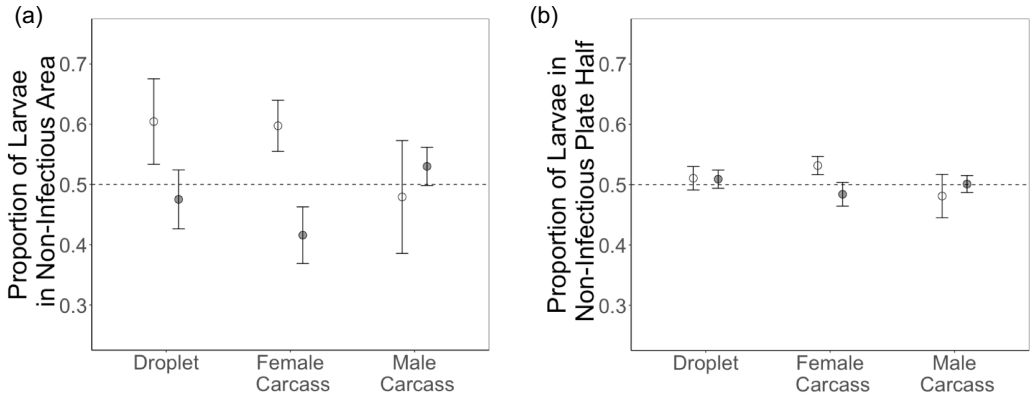


Figure 2 – Larval foraging choice. Mean±SE proportion of larvae on choice plates after 72 hours found (a) within area 2.2cm in diameter of the non-infectious food source and (b) on the non-infectious food source's half of the plate. Results from both choice (white points) and control plates (grey points) are shown. In the case of control plates, where only non-infectious food sources are present, the mean±SE is derived from the proportion of larvae present at a randomly selected side of the plate. Food sources included droplets of TRIS, a male carcass or female carcass.

DCV is transmitted to larvae when scavenging on infected carcasses

DCV was detected in larvae collected from plates containing an infected carcass (Figure 3a, Table 1), confirming that scavenging infected carcasses is a viable route of virus transmission. As expected, larvae surrounding DCV-infected carcasses were found to have significantly higher DCV titres when compared to larvae collected from control plates (which contained only uninfected carcasses). However, we also detected DCV infection in larvae surrounding clean carcasses that were housed in a two-choice plate (containing both infected and uninfected carcasses) (Figure 3a), suggesting that some larvae may have moved between food sources in these plates during the assay.

No effect of virus acquisition on larval development

Acquiring infection by scavenging on infectious carcasses had no detectable effect on larval development into pupae (Figure 3b), or in the proportion of pupae that eclosed as adults (Figure 3c; Table 1). However, more larvae developed to pupal stage when they fed on a female carcass (Figure 3b; Table 1): 50% of larvae feeding on female carcasses reached pupation, while a significantly lower proportion (32%)

reached pupation if they had fed on male carcasses (Figure 3b). Following pupation, there was no effect of carcass sex or infection status on the proportion of pupae that eclosed as adults (Figure 3c, Table 1– Figure S2; Appendices 7.1.1).

Virus acquired during the larval stage can persist into adulthood

We measured DCV titres in flies that eclosed as adults (Figure 3d). While no DCV infection was detected in flies originally collected near clean carcasses, we detected DCV in 9 out of 15 adult flies that were collected from infected carcasses, suggesting that DCV infection can persist through metamorphosis into the adult insect stage.

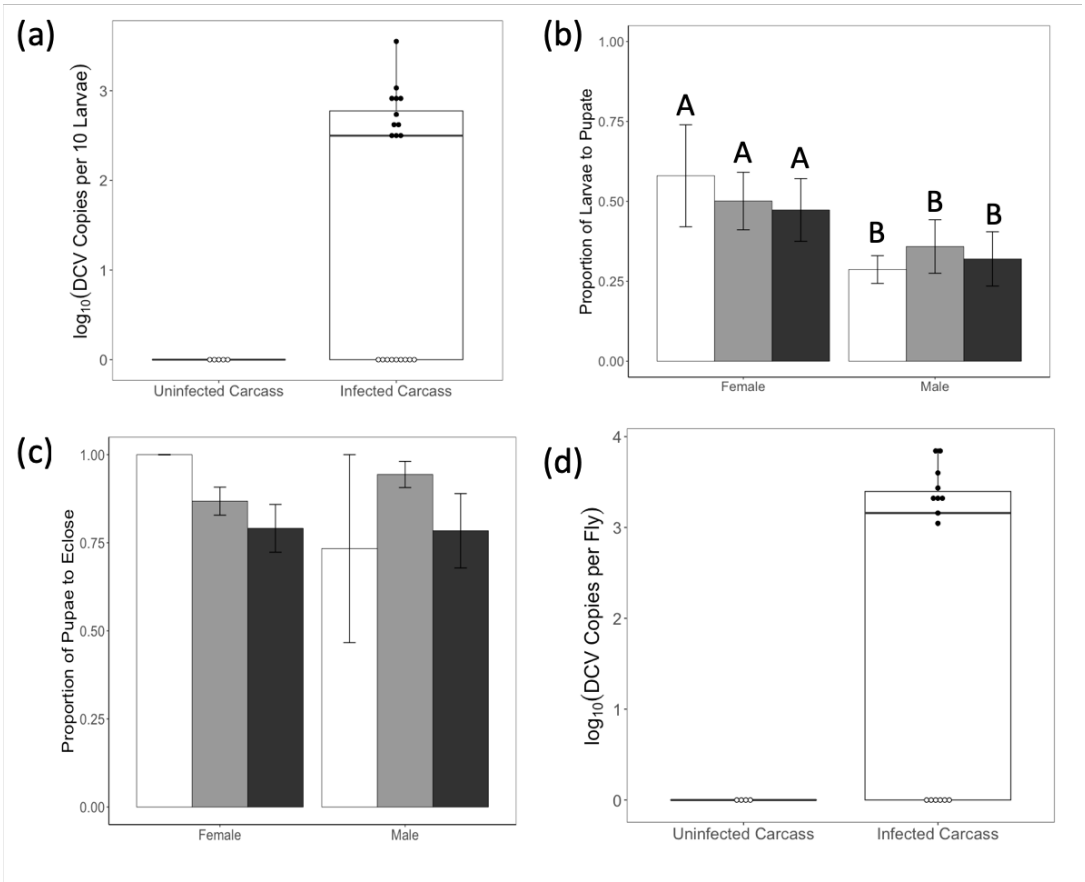


Figure 3. Fitness consequences of infectious scavenging. (a) The number of DCV copies present in larvae, quantified immediately after choice assays having fed on an uninfected carcass on a control plate or a choice plate and an infected carcass from a choice plate. Mean±SE proportion of larvae taken from carcass sites on both choice and control plates to (b) pupate and (c) eclose. Larvae (and the subsequent pupae) were taken from male and female carcasses and varied in their infectious status, an uninfected carcass on a control plate (white bar), an uninfected carcass on a choice plate (grey bar) or an infected carcass on a choice plate (black bar). (d) The number of DCV copies present in adults derived from choice plate assays.

DCV infection increases fecundity

In addition to measuring DCV avoidance by the number of eggs laid, we measured the total number of eggs laid over the course of the 48 hours. Infected mothers laid significantly more eggs than healthy mothers (Figure 4a; Table 2).

Response	Predictor	DF	F	p-value
Larval Foraging Choice by Plate Half	Carcass Sex/TRIS	2	0.599	0.741
	Carcass Infection	1	0.632	0.426
	Carcass Sex/TRIS * Carcass Infection	2	2.76	0.251
Larval Foraging Choice by Carcass Area	Carcass Sex/TRIS	2	0.512	0.774
	Carcass Infection	1	3.60	0.0579
	Carcass Sex/TRIS * Carcass Infection	2	4.50	0.106
Larval DCV Titre	Carcass Sex	1	0.697	0.404
	Carcass Infection	2	6.42	0.0403*
	Carcass Sex * Carcass Infection	2	0.218	0.897
Adult DCV Titre	Carcass Infection	2	9.67	0.0079**
Number of Larvae to Pupate	Carcass Sex	1	13.3	0.0003***
	Carcass Infection	2	0.0745	0.963
	Carcass Sex * Carcass Infection	2	0.618	0.734
Number of Pupae to Eclose	Carcass Sex	1	0.0174	0.895
	Carcass Infection	2	0.180	0.914
	Carcass Sex * Carcass Infection	2	0.149	0.928

Table 1. Model outputs for statistical tests performed on all experiments testing the causes and costs of infection avoidance in *D. melanogaster* larval foraging. Significant predictors are marked with asterisks ($p<0.05$=*, $p<0.01$=** and $p<0.001$=***).

Oviposition preference changes over time and depends on the female's infection status

Female flies showed a clear preference for oviposition sites containing a carcass, but this choice depended on the fly infection status (Figure 4b; Table 2). The 3-way interaction between time, oviposition site, and the mother's infection status was highly significant (Figure 4b & 4c; Table 2). This means that the oviposition sites

where mothers laid their eggs changed over time in a manner dependent on the mother's infection status. Within the first 24-hour period, uninfected female flies laid significantly more eggs at sites containing a clean carcass compared to sites with an infected carcass or just food (Figure 4b). Female flies infected with DCV, however, did not distinguish between infected and clean carcasses, but still laid significantly fewer eggs at sites without any carcass (Figure 4b). In the 24-48-hour observation period, uninfected females still laid more eggs at sites with carcasses, but no longer preferred the sites containing a clean carcass (Figure 4c; Table 2). DCV-infected females also laid more eggs at sites with an uninfected carcass (pairwise contrast, $p<0.0001$), but laid even more eggs on sites containing an infected carcass (pairwise contrast, $p<0.001$) (Figure 4c).

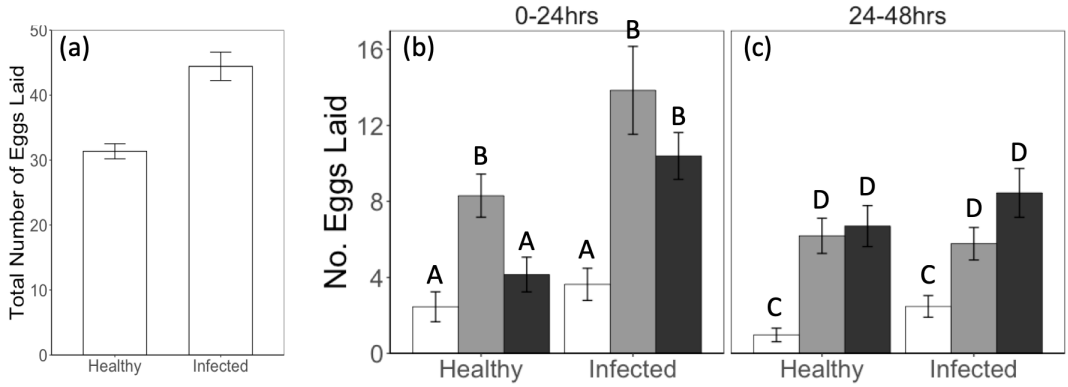


Figure 4. Adult oviposition choice. The mean±SE number of eggs laid by infected and uninfected mothers (a) at the end of the 48-hour laying period in a single oviposition arena and at the three oviposition sites after the (b) first 24 hours of the experiment and (c) second 24-hour period. Oviposition sites use the same colour scheme: food only oviposition sites in white, food and uninfected carcass sites in grey and food and infected fly carcass sites in black.

Fitness consequences of oviposition preference

Egg-to-adult viability differed significantly between oviposition sites and was lower in food-only sites compared to sites containing a carcass (Figure 5a; Table 2). Clutches emerging at carcass sites however, did not differ in their egg-to-adult viability (Figure 5a; Table 2), even though we detected significantly more DCV in flies that developed around DCV-infected carcasses (Figure 5b). The infection status of mothers did not affect egg-to-adult viability (Figure 5a; Table 2) on the viral load of these clutches (Figure 5b; Table 2).

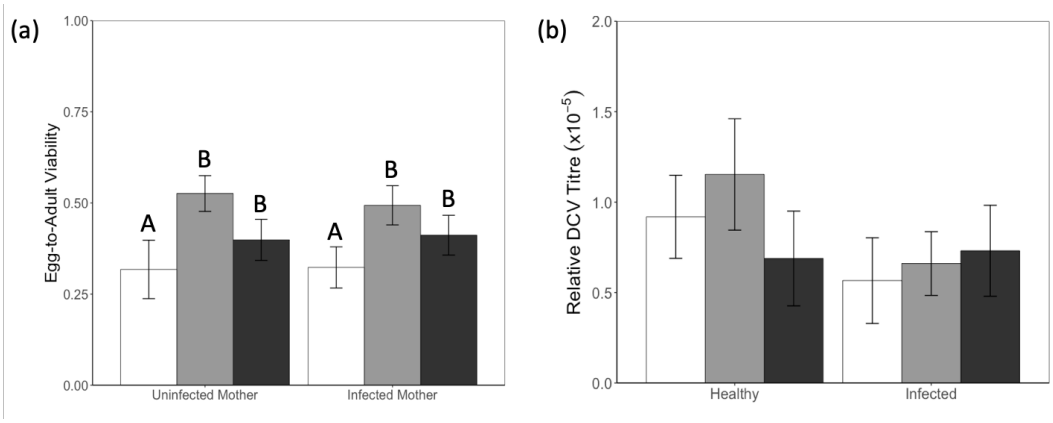


Figure 5. Adult oviposition fitness consequences. The mean ± SE (a) proportion of eggs to develop through to adulthood (egg-to-adult viability) of the clutches laid during the oviposition site choice assay and (b) the ratio of viral RNA to fly RNA (x10^{-5}) in clutches laid during the oviposition site choice assay. Oviposition sites use the same colour scheme: food only oviposition sites in white, food and uninfected carcass sites in grey and food and infected fly carcass sites in black.

Response	Predictor	Df	F	p
Total Eggs Laid 0-48hrs	Mother Infection	1	26.6	<0.0001***
Number of Eggs Laid	Time	1	0.0702	0.79
	Ovi. Site	2	212	<0.0001***
	Mother Infection	1	0.0315	0.86
	Time * Ovi. Site	2	29.8	<0.0001***
	Time * Mother Infection	1	0.0947	0.76
	Ovi. Site * Mother Infection	2	7.37	0.0081**
	Time * Ovi. Site * Mother Infection	2	10.5	<0.0001***
Egg-to-Adult Viability	Ovi. Site	2	5.61	0.0053**
	Mother Infection	1	0.0128	0.88
	Ovi. Site * Mother Infection	2	0.528	0.592
Clutch DCV Load	Ovi. Site	2	2.55	0.0988
	Mother Infection	1	0.628	0.436
	Ovi. Site * Mother Infection	2	1.46	0.252

Table 2. Model outputs for statistical tests performed on all experiments testing the causes and costs of infection avoidance in *D. melanogaster* adult oviposition. Significant predictors are marked with asterisks (p<0.05=*, p<0.01=** and p<0.001=***).

2.4 Discussion

Viral infection is widespread among invertebrates (Shi et al., 2016; Webster et al., 2015), and can cause considerable morbidity and mortality (Arnold et al., 2013; Escobedo-Bonilla et al., 2008; Gupta et al., 2017; Wilfert et al., 2016). We should therefore expect selection for mechanisms that allow hosts to detect and avoid infectious conspecifics or potentially infectious environments (Curtis, 2014; Kiesecker et al., 1999). In the present work, we examined how larval foraging and adult oviposition in *D. melanogaster* are modified in the presence of potential infection by the horizontally transmitted *Drosophila* C virus (DCV), which is known to cause a variety of physiological and behavioural pathology in fruit flies (Arnold et al., 2013; Gupta et al., 2017; Stevanovic and Johnson, 2015; Vale and Jardine, 2015).

Our results confirm previous findings that *Drosophila* larvae will actively cannibalize conspecific carcasses when placed in a nutrient-poor environment (Ahmad et al., 2015; Vijendravarma et al., 2013), and go further to demonstrate that necrophagy is a viable route for transmission of *Drosophila* C Virus. The consumption of infectious conspecifics, either through cannibalism or necrophagy, has been demonstrated as a viable route of infection in a wide range of mammalian, amphibian and insect species (Alpers, 2008; Forbes, 2000; Pearman et al., 2004; Qureshi et al., 2000; Williams and Hernández, 2006). In holometabolous insects, this phenomenon has been particularly well investigated in Lepidoptera, where cannibalism and/or necrophagy of infected conspecifics has also shown to be a viable route of transmission of several viruses during larval development (Boots, 1998; Dhandapani et al., 1993; Elvira et al., 2010; Vasconcelos, 1996; Williams and Hernández, 2006).

Despite the risk of acquiring infection during cannibalistic foraging, we found no evidence that larval-stage flies could discriminate and avoid infectious carcasses from clean ones. Our findings contrast with a recent study in which *Drosophila* larvae showed avoidance of food contaminated with a bacterial suspension of virulent *Pseudomonas entomophila* (Surendran et al., 2017). In the same study, avoidance

was no longer observed when using a less virulent strain of the bacterial pathogen, suggesting that external cues about the relative risk and severity of infection are key to avoidance behaviours (see also (Vale and Jardine 2016). The differences in findings likely result from different olfactory and chemo-sensory factors involved in viral and bacterial detection in *Drosophila* larvae. Furthermore, while Surendran et al (2017) tested evasion in 1st instar larvae, we investigated larval foraging choice during the 3rd instar, as this is the period of development when foraging activity and feeding is known to peak (Sokolowski, 2001). Given that larvae are known to actively migrate towards higher quality food (Durisko and Dukas, 2013), the lack of trophic infection avoidance suggests that selection for avoidance of this viral infection is weak. Weak selection for avoidance would be expected if, for example, the fitness costs of DCV infection are low during larval stage infection.

Our data is consistent with a low cost of infection in larvae, as the low titres of DCV acquired during larval feeding on infected carcasses did not have severe consequences for larval development. Our results contrast with a previous study on DCV infection of larval *D. melanogaster* which reported a 14% reduction in egg-to-adult viability, and severe mortality in adults emerged from infected larvae (Stevanovic and Johnson 2015). Unlike the relatively natural route of pathogen exposure employed in our work, larva in that study were exposed to a highly-concentrated homogenate of DCV-infected flies and exposed continuously during development until 4-days post-eclosion. This difference in viral exposure may explain the more severe costs of DCV infection compared to this study.

In contrast to the lack of discrimination seen during larval foraging, we found that adult female flies do discriminate between different types of oviposition sites. Uninfected female flies laid more eggs on sites containing an uninfected or infected carcass and food, than a site comprised only of food despite the infection risk this presents. Preference for carcass-containing sites could be explained by flies preferring to lay eggs on sites with irregular surfaces, however as uninfected mothers avoid infected carcass sites, it is more likely a result of conspecific

carcasses offering additional nutrition that undermines or negates the infection risk they pose (Albeny-Simoes et al., 2014). Starved *D. melanogaster* larvae assess the nutritional value of carcasses, ranging from conspecifics to natural predators (Ahmad et al., 2015), and tune their foraging strategies accordingly to optimally forage. Clutches developing on oviposition sites with a carcass present had significantly higher egg-to-adult viability than food only sites despite their significantly greater larval density (Figure 5a). The preference we see for oviposition sites containing a carcass may therefore indicate that the nutritional value of carcasses on the oviposition sites, rather than infection risk, is a greater driver of oviposition-site preference.

During the first 24 hours of egg laying, uninfected flies laid significantly more eggs around uninfected carcasses. This suggests that the presence of DCV is being detected and avoided during oviposition. It is unclear which cues of DCV are detected by females, whether they are detecting the virus directly or cues of virus derived pathology in the fly carcass. Similar avoidance of pathogenic bacteria has been described in both *D. melanogaster* (Babin et al., 2014; Kurz et al., 2017; Stensmyr et al., 2012) and *C. elegans* (McMullan et al., 2012; Meisel and Kim, 2014). Avoidance of virus infection has also been described in a range of invertebrates, such as gypsy moth larvae that avoid eating leaves contaminated with virus (Parker et al., 2010) and lobsters that avoid virus-infected conspecifics (Behringer et al., 2006). This avoidance likely relies on dedicated chemosensory pathways for olfactory cues (Kurz et al., 2017; McMullan et al., 2012; Meisel et al., 2014; Stensmyr et al., 2012).

In the 24-48-hour period, the preference for uninfected carcasses was not observed (Figure 4c). We interpret this shift in oviposition-site preference as the result of a trade-off faced by females between minimizing DCV infection risk and maximizing fecundity. The finite nutritional value of each oviposition site dictates an optimal clutch size that each site can support. If females exceed this, fewer resources are available per offspring. As uninfected flies laid more eggs on non-infectious carcass

sites in the first 24 hours, the optimal clutch size is approached sooner than the other two sites. Fruit flies integrate the nutritional quality of oviposition sites into deciding between laying more eggs and acquiring more resources to develop more eggs (Lihoreau et al., 2016b), a trade-off that is also seen in a range of other organisms (Albeny-Simoes et al., 2014; Blaustein, 1999; Lihoreau et al., 2016b; Tjørnløv et al., 2015). In order to maximize the number of eggs laid, females therefore appear to risk DCV infection by laying their eggs near an infected carcass. The relative nutritional value and the potential costs of DCV infection are patent in the egg-to-adult viability of offspring from each oviposition site: the increase in viability between the food-only site and both the uninfected and infected carcass sites reflects the nutritional difference between these sites. Figure 5a suggests the benefits of oviposition near any carcass appear to outweigh the potential costs of virus infection.

In contrast to uninfected females, females infected with DCV did not discriminate between infectious and non-infectious carcasses, laying the same number of eggs in either oviposition site (Figure 4b, c). Furthermore, in the second 24-hour period, infected females laid significantly more eggs at infectious carcass sites. We interpret this difference in discrimination between infected and healthy females as being driven by the mother's, rather than the offspring infection risk. For infected females already paying the cost of infection, there is little benefit to avoiding infectious sites.

In summary, our results show that *D. melanogaster* larvae and adults respond to infection risk differently during foraging and oviposition. Notably, oviposition site choice was affected by the female's infection status and the time-dependent nutritional value of oviposition sites. The initial DCV avoidance shown by mothers during oviposition may also explain why larvae do not avoid DCV during foraging. Alongside a relatively low cost of infection, larvae simply may not need to avoid infection because their mothers have evolved to avoid infectious sites where possible during oviposition. As larvae are not able to forage over large distances, their development - and ultimately their fitness - relies heavily on their mother's

capacity to pick the environment that maximizes nutritional value while minimizing the risk of infection.

Chapter 3: Infection Causes Sex-Specific Changes in Social Aggregation Behaviour

I am the sole author of this text, with comments on earlier drafts from Pedro Vale.

Siva-Jothy, J. A., Vale, P. F. (2019) Viral infection causes sex-specific changes in fruit fly social aggregation behaviour. *bioRXiv*. 630913.

Abstract

Host behavioural changes following infection are common and could be important determinants of host behavioural competence to transmit pathogens. Identifying potential sources of variation in these behaviours is therefore central to our understanding of disease transmission. Here, we test how group social aggregation and individual locomotor activity vary between different genotypes of male and female fruit flies (*Drosophila melanogaster*) following septic infection with *Drosophila* C Virus. We confirm previously reported genetic-based variation in both locomotor activity and social aggregation but did not detect a significant effect of DCV infection on fly activity or sleep patterns within the initial days following infection. However, DCV infection caused sex-specific effects on social aggregation, as male flies in most genetic backgrounds showed a general tendency to increase the distance to their nearest neighbour when infected. We discuss potential causes of these differences in the context of individual variation in immunity and relate them to individual variation in disease transmission.

3.1 Introduction

Parasitism is one of the most successful ecological relationships among living organisms (Dobson et al., 2008; Schmid-Hempel, 2011; Weinstein and Kuris, 2016). One reason for this success may be due to the many ways parasites and pathogens can modify host physiology and behaviour (de Roode and Lefèvre, 2012; Hart, 2011; Kazlauskas et al., 2016; Poulin, 2013; Vale et al., 2018). While changes to host physiology, and immunity in particular, following infection are well known, it is striking how many animals experience similar behavioural changes following infection (Hart, 1988; Poulin, 1995). Common behavioural responses to infection include eating and moving less, as well as foregoing social and sexual interactions (Hart, 2011, 1988; Kazlauskas et al., 2016; Lopes, 2014). Whether host behavioural changes in response to infection are evolved host responses, parasite manipulations, or a coincidental by-product of infection, they have potentially important consequences for disease transmission (Barron et al. 2015). This is particualry clear for behaviours such as individual locomotor activity or group social aggregation, which will directly determine how frequently individuals interact. This is central to disease transmission because contact rates between susceptible and infected individuals are one of its key determinants. Assessing how host behaviours that influence contact rates might change following infection is therefore central to understanding the spread of infectious disease.

The extent to which host behaviours are modified during infection is likely to depend on genetic and environmental factors. Even in the absence of infection, individuals of some genetic backgrounds are more likely to aggregate than others (Anderson et al., 2016; Saltz, 2011), while males and females in a broad range of host species often exhibit distinct behavioural profiles (Manoli et al., 2005; Walum et al., 2008). How these different sources of variation influence infection-induced behavioural changes is relatively understudied (Barron et al., 2015). Measuring how males and females of different genetic backgrounds modify their behaviour during infection

may highlight groups of individuals with higher contact rates and offer insight into the potential causes of heterogeneity in pathogen spread.

Testing if locomotor and aggregation behaviours change following infection, and if these changes differ between genetic backgrounds, is not straightforward for most host species. It requires knowledge of how individuals within a population differ in their genetic backgrounds and the ability to expose many individuals of the same background to infection in controlled conditions, while comparing their behavioural responses to infection to individuals of the same background that do not experience infection. For many animal species, and certainly in human populations, this type of intervention is either extremely challenging or not feasible. One alternative is to leverage the tools offered by model systems. *Drosophila melanogaster*, for example, has been widely used as a model system for behavioural genetics (Dubnau, 2014; Sokolowski, 2001), and used specifically to study social aggregation and locomotor activity (Pfeiffenberger et al., 2010; Saltz, 2011; Simon et al., 2012). Further, *D. melanogaster* is a powerful model of immunity in response to a range of bacterial and viral pathogens (Troha and Buchon, 2019). Previous work has shown that *D. melanogaster* exhibits a range of behavioural changes following *Drosophila* C Virus (DCV) infection, including pathogen avoidance during oviposition (Siva-Jothy et al., 2018), and foraging (Vale and Jardine, 2016). Here, we test whether DCV infection changes social aggregation and locomotor activity in *D. melanogaster*, and if these effects vary with genetic background and sex.

3.2 Materials & Methods

Flies & Rearing Conditions

We used males and females from 10 lines sourced from the *Drosophila* Genetic Resource Panel (DGRP) (Mackay et al., 2012), and are among the most and least susceptible genetic backgrounds to systemic *Drosophila* C Virus infection (Magwire et al., 2012). Flies were reared in plastic vials on a standard diet of Lewis medium at 18±1˚C with a 12 hour light:dark cycle with stocks tipped into new vials every 14

days. One month before the experiment, flies were transferred to incubators and maintained at 25±1˚C with a 12 hour light:dark cycle at low density (~10 flies per vial) for two generations.

Virus Culture & Infection

The *Drosophila* C Virus (DCV) isolate was originally isolated in Charolles, France (Jousset et al., 1977) and the stock used in this experiment was grown in Schneider *Drosophila* Line 2 (DL2) as previously described (Vale and Jardine, 2015c) diluted ten-fold (10^8 infectious units per ml) in TRIS-HCl solution (pH=7.3), aliquoted and frozen at -70˚C until required. To infect with DCV, 3-5 day old flies were pricked in the pleural suture with a 0.15mm diameter pin, bent at 90° ~0.5mm from the tip, dipped in DCV (or TRIS-HCl for controls).

Measuring Drosophila Activity

Fly activity was measured using the *Drosophila* Activity Monitor (DAM2 System, TRIKinetics), in an incubator maintained at 25°C in a 12:12 light:dark cycle (Pfeiffenberger et al., 2010). Single flies were either systemically infected or control pricked as described above, and immediately placed in a single DAM tube and allocated a random slot in one of 8 DAM monitor units (each unit is capable of housing a maximum of 32 tubes). At least one slot of each monitor unit was left empty and another contained an empty tube, as negative controls. While flies were monitored continuously for 4 complete days. Flies that died during this 4-day period were removed from the dataset.

Activity data and statistical analyses

Raw activity data was processed using the DAM System File Scan Software (Pfeiffenberger et al., 2010), and the resulting data was manipulated using Microsoft Excel. Activity counts for each individual fly were combined into 5-minute bins. We analysed fly activity data using three metrics: total locomotor activity, proportion of time spent asleep and the average activity when awake (Vale and Jardine, 2015c). Total locomotor activity refers to the sum of all recorded movements during the 4-

day measuring period and is an outcome of how often a fly sleeps and how much it moves during bouts of awake activity. In *Drosophila*, sleep is defined as five minutes of continuous inactivity, sharing several features with mammalian sleep, such as being followed by an increased arousal threshold, and being regulated independently from the circadian clock (Shaw et al., 2000). To assess the proportion of time spent asleep, we used the proportion of all 5-min bouts (n=1152) where no activity was logged. To quantify awake activity, we calculated the average level of locomotor activity across every 5-min period where at least one instance of movement was recorded. Average activity when awake can help characterise lethargy when individuals are active, an important behavioural symptom of infection (Hart, 1988).

Measuring social aggregation

Social aggregation was measured in a separate experiment, by calculating the nearest neighbour distance (NND) between each individual fly within a group (Anderson et al., 2016; Clark and Evans, 1954; Simon et al., 2012). The experiment was conducted over five experimental blocks, each carried out over a single day, where each genetic background, sex and infection treatment was measured. In total, we measured social aggregation in 580 groups of flies, with n=14-16 groups per genetic background, sex and infection status combination. Social aggregation was measured in 55mm Petri dishes with 2% agar poured in until 3mm from the lid in order to limit flight. Flies were pricked with DCV or TRIS as described above and, under light CO_2 anaesthesia, transferred to Petri dishes in groups of twelve. Due to reducing anaesthesia as much as possible to curtail behavioural defects associated with over-exposure to CO_2 (Colinet and Renault, 2012), and experimenter error, some flies escaped Petri dishes before they were closed. A total of 448 dishes contained twelve flies, while 113 and 19 contained eleven and ten, respectively. Flies within a Petri dish were the same genetic background, sex and infection treatment. Once transferred, flies were left in Petri dishes to acclimate for 30 minutes. This acclimation period was identified in a prior experiment where it was observed that after 30 minutes, fly movement in arenas was minimal, as shown previously

(Anderson et al., 2016; Simon et al., 2012). A single image was recorded of each Petri dish using a 13 Megapixel camera, followed by a second image (10-20 minutes later). Using these images we calculated the NND using ImageJ software (Schneider et al., 2012), by marking flies in the centre of their thorax with the multi-point tool. We calibrated the distance between flies in photos using the 55mm width of the Petri dish and calculated the nearest neighbour distance between each pair of flies in millimetres using the 'NND' package in ImageJ. These values were used to calculate the median NND for each petri dish (Anderson et al., 2016; Clark and Evans, 1954). To account for differences in body lengths between genetic backgrounds and sexes, we also calculated the NND using body lengths by dividing millimetre distances by the mean body length of a randomly selected group of 30-40 individuals from each genetic background and sex combination.

Statistical Analysis

We tested if differences in locomotor activity and social aggregation could be attributed to fly genetic background or sex. Data from both experiments were analysed using Generalised Linear Models (GLMs). Models used a full factorial 3-way interaction between infection status (control /infected), sex (male / female) and DGRP line (10 genetic backgrounds), all modelled as fixed effects. Analysis of social aggregation used a model listing only the median nearest neighbour distance of each dish as its response variable. To assess locomotor activity, we analysed 3 response variables in separate GLMs (total activity, proportion of time asleep, awake activity), adjusting the significance threshold to 0.01667 using Bonferroni correction to account for multiple-testing. The effect of experimental block was originally incorporated into our statistical analysis as a random effect in a Generalised Linear Mixed Model (GLMM), however it was removed as it accounted for no residual variance. All statistical analyses and graphics were carried out and produced in R 3.3.0 using the *ggplot2* (Wickham, 2016) and *lme4* (Bates et al., 2015) packages.

3.3 Results

Social aggregation

We found a significant effect of genetic background in the median nearest neighbour distances (NND), suggesting that there is significant genetic variation in this measure of social aggregation (Figure 1; Table 1). We found no evidence of sexual dimorphism in social aggregation across multiple genetic backgrounds, with no significant interaction between sex and genetic background. However, we observed that while female aggregation was not affected by infection, infected males aggregated further apart from each other compared to uninfected males (Figure 1; Table 1). This increase in the NND following infection was generally observed in males, regardless their genetic background (Figure 1; Table 1). We also detected an expected sexual dimorphism in body size, where female *D. melanogaster* were typically larger than males (Figure S1; Table S1 - Appendices 7.2.1). Incorporating this size difference into measures of social aggregation, by measuring body lengths between individuals did not alter the results (Figure S2; Table S2 - Appendices 7.2.1).

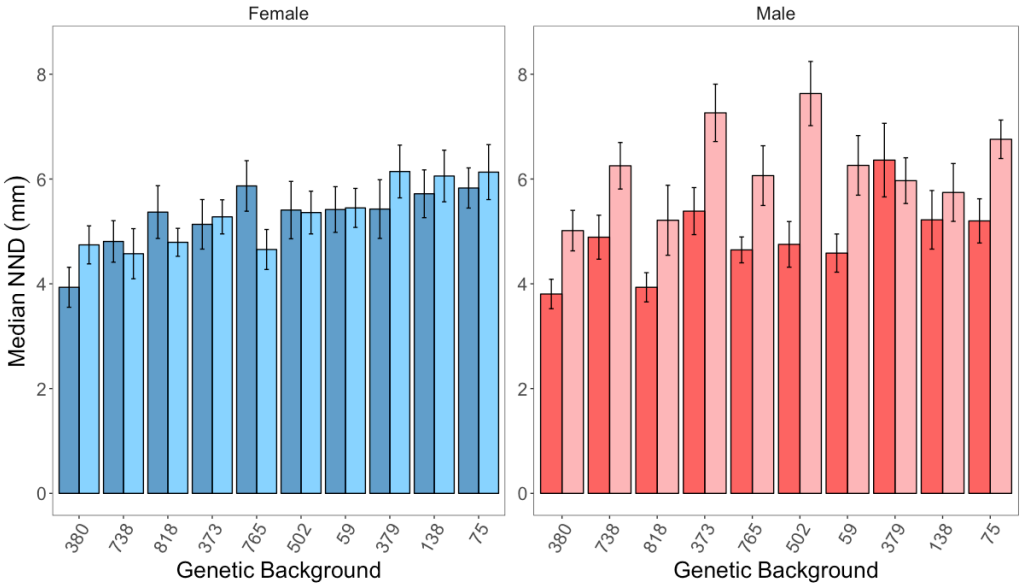


Figure 1 – Mean±SE median nearest neighbour distance (NND) in millimetres (mm) of adult flies placed in Petri dishes for at least 30 minutes until settled. (a) Uninfected female-only arenas shown in blue, and infected female-only arenas in pale blue. (b) Uninfected male-only arenas are shown in red, and infected male-only arenas in pink. The x-axis of both panels is ordered from the lowest to highest mean median NND of female flies of a single genetic background.

Response	Predictor	Df	F	p
Median NND	Genetic Background	9	5.0249	<0.0001
	Sex	1	2.7870	0.13
	Infection	1	21.1301	<0.0001
	Genetic Background * Sex	9	1.4112	0.19
	Genetic Background * Infection	9	0.9654	0.49
	Sex * Infection	1	19.6600	<0.0001
	Genetic Background * Sex * Infection	9	1.6729	0.12

Table 1 - Model outputs for statistical tests performed on social aggregation, testing the causes of variation in sociality in males and females of 10 *D. melanogaster* genetic backgrounds. Significant predictors are marked with asterisks (p<0.05=*, p<0.01=** and p<0.001=***).

Locomotor activity

All three parameters of total locomotor activity, the proportion of time spent asleep and the average activity when awake, were affected by a combination of sex and genetic background (Figures 2 and 3; Table 2). However, there was no detectable difference in how much infected and healthy flies moved or slept, and hence no evidence that infection impacted on any parameter of fly locomotor activity (Figures 2 and 3; Table 2).

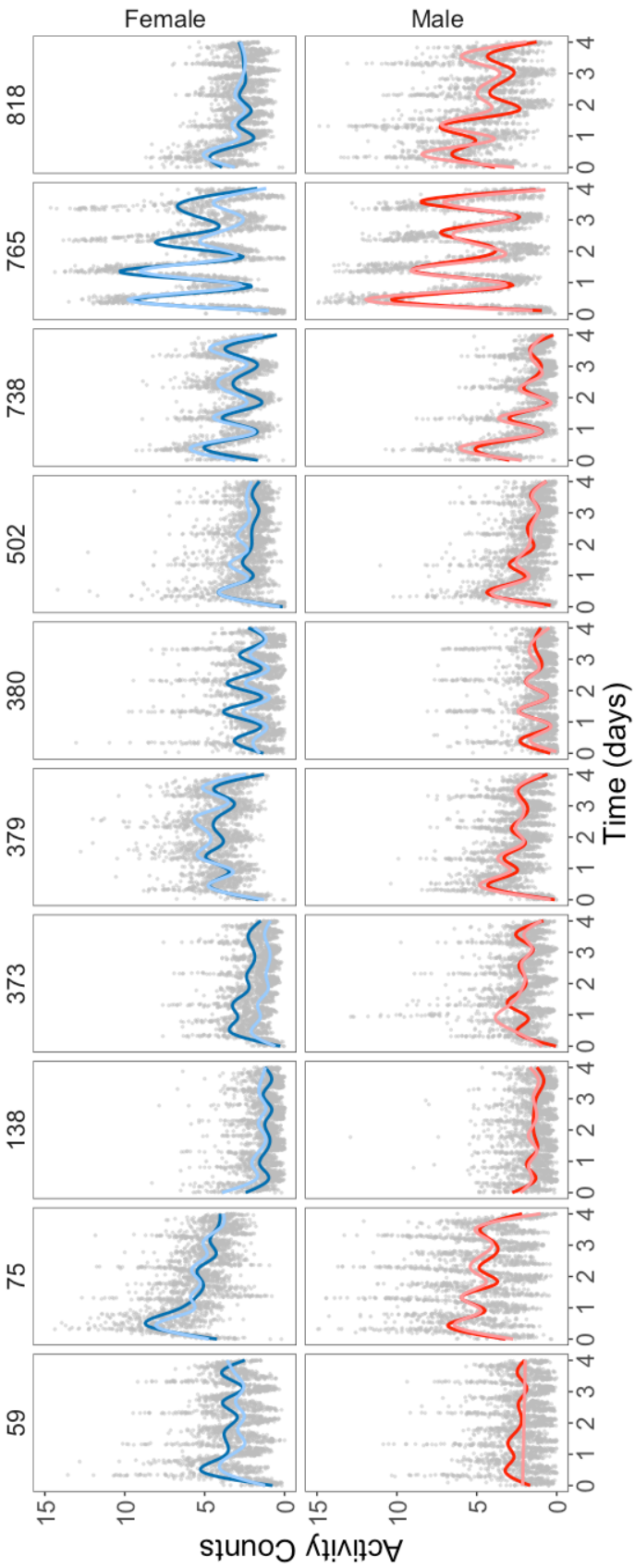


Figure 2. Activity counts of adult flies for the first 4 days locomotor activity was measured in the DAM. The mean activity counts of DAM tubes containing single flies of the same sex and DCV infection status are represented by generalised additive model lines where uninfected females are shown in blue, infected females in pale blue, uninfected males are shown in red, and infected males in pink.

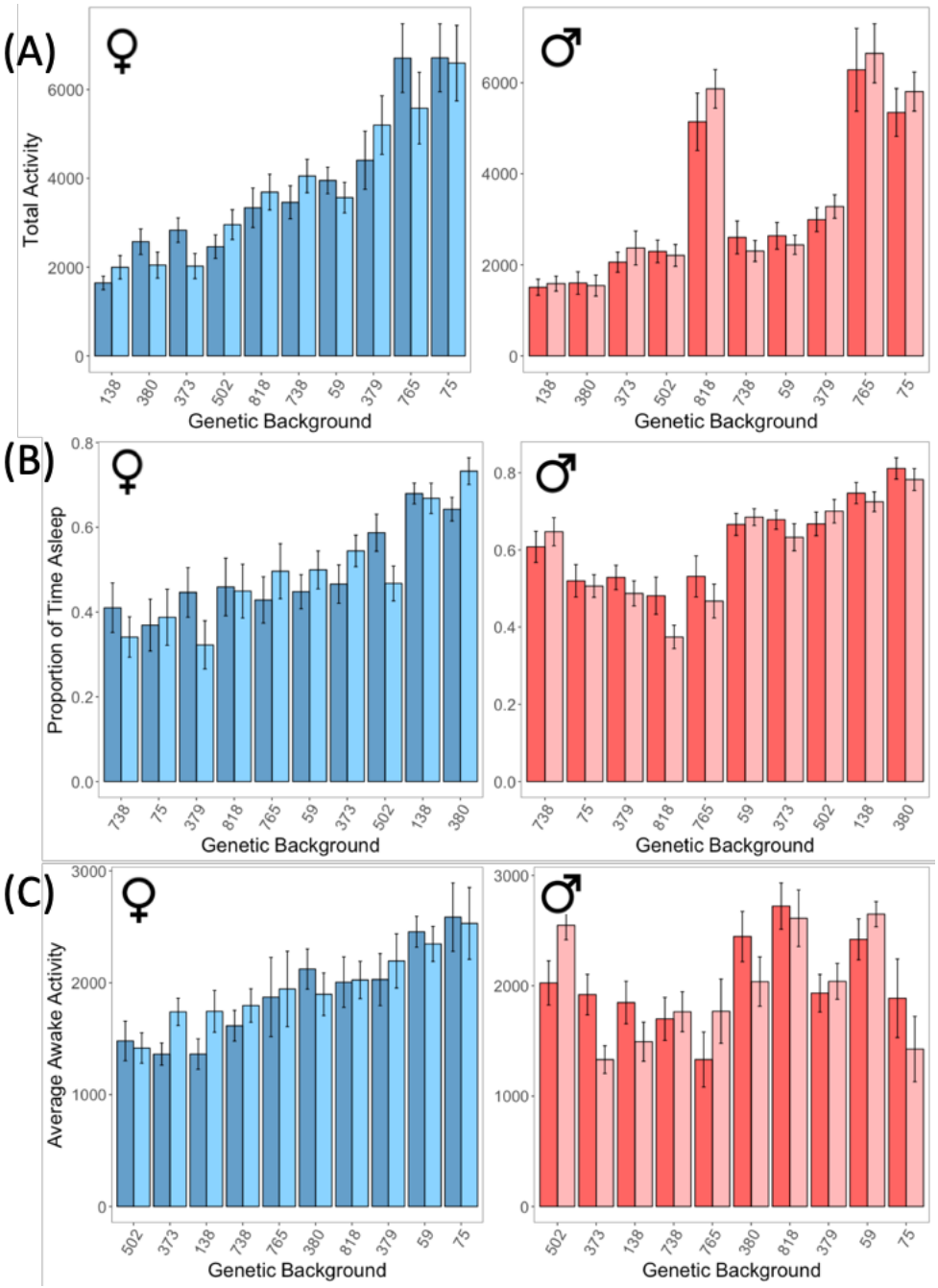


Figure 3 – Mean±SE (A) total locomotor activity, (B) proportion of time flies spent sleeping and (C) mean activity while flies were awake, during the first 96 hours of DCV infection. Across all panels, sex and infection status are represented by colour with uninfected females shown in blue, infected females in pale blue, uninfected males in red, and infected males in pink. The order of genetic backgrounds on the x-axis of each of panel follows the ascending order of female flies.

Response	Predictor	Df	F	p
Total Activity	Genetic Background	9	14.83	<0.0001***
	Sex	1	1.537	0.21
	Infection	1	0.117	0.73
	Genetic Background * Sex	9	3.0485	0.0013*
	Genetic Background * Infection	9	1.4125	0.18
	Sex * Infection	1	3.9707	0.047
	Genetic Background * Sex * Infection	9	1.9471	0.043
Proportion of Time Spent Asleep	Genetic Background	9	25.1759	<0.0001***
	Sex	1	77.9823	<0.0001***
	Infection	1	0.6939	0.41
	Genetic Background * Sex	9	3.444	<0.001**
	Genetic Background * Infection	9	0.8021	0.61
	Sex * Infection	1	0.7513	0.39
	Genetic Background * Sex * Infection	9	1.4612	0.16
Awake Activity	Genetic Background	9	8.1673	<0.0001***
	Sex	1	0.6641	0.54
	Infection	1	0.0008	0.86
	Genetic Background * Sex	9	5.2153	0.0013*
	Genetic Background * Infection	9	0.8716	0.58
	Sex * Infection	1	0.8430	0.44
	Genetic Background * Sex * Infection	9	1.2998	0.61

Table 2 – Model outputs for statistical tests performed on host activity data, testing the causes of variation in locomotor activity, sleep patterns and average awake activity in males and females of 10 D. melanogaster genetic backgrounds. Significance thresholds are corrected for multiple testing using Bonferroni correction, with significant predictors are marked with asterisks ($p<0.01667$=*, $p<0.001$=** and $p<0.0001$=***).

3.4 Discussion

We observed that how closely flies aggregate with one another differs with their genetic background. The genetic variation we observed is similar to other studies that have measured nearest neighbour distance (Anderson et al., 2016), as well as other aspects of *Drosophila* social behaviour, such as group size preference (Saltz, 2011) and group composition (Philippe et al., 2016). Group composition is affected by the natural *foraging* gene polymorphism, where larvae are either sitters, which aggregate toward conspecifics at food sources or rovers, who are more prone to independent food searching behaviour. Larger groups of larvae on food patches are more likely to be comprised of sitters, as rovers leave food patches after overcrowding (Philippe et al., 2016). Genetic components of social behaviour have also been identified in a number of mammal species, including humans (Anacker and Beery, 2013; Feldman et al., 2016). In a number of vole species, variation in oxytocin (Parker et al., 2001; Ophir et al., 2012) and arginine vasopressin (Winslow et al., 1993) receptor density is associated with between-species variation in pair-bonding and monogamy. Oxytocin receptor density has even been shown to within-species variation in pair-bonding, changing seasonally alongside sociality in female meadow voles (Parker et al., 2001).

Overall, our results indicate a significant sex difference in the effect of infection on social aggregation but no effect of infection on locomotor activity in either sex. While healthy male and female aggregation did not differ, once infected, males aggregated further apart, while female aggregation did not change. One possible explanation for why males aggregate further apart following infection is a sex difference in immunity and the costs of social aggregation (Kelly et al., 2018). Sexually dimorphic immunity may be particularly relevant as male *D. melanogaster* exhibit a stereotyped suite of aggressive behaviours (Baxter et al., 2015; Dierick and Greenspan, 2006; Dierick, 2007). While fighting can gain males access to valuable resources, it often incurs substantial costs (Chen et al., 2002; Lim et al., 2014). DCV infection could exacerbate the cost of male aggregation, as resources would also

need to be spent on fighting infection, which could lead to males aggregating less. Despite females also fighting one another, this aggression is generally less costly (Nilsen et al., 2004; Ueda and Kidokoro, 2002). Females may therefore still be able to aggregate relatively closely while fighting DCV infection.

Irrespective of the metric used, we found no measurable effect of DCV infection on locomotor activity. Other work has shown decreases in *Drosophila* daily movement following injection with DCV, where a marked reduction is seen after 4 days of infection (Arnold et al., 2013). Reduced daily locomotor activity has also been observed in *Drosophila* after 3 days of infection with the DNA virus Kalithea virus (Palmer et al., 2018). Injecting, rather than pricking, flies with viral suspension, allows more precise control of infectious dosage, which could also increase infection severity (Neyen et al., 2014). Another potential explanation is that we infected flies via thoracic pricking, as opposed to abdominal injection which has been shown to reduce resistance to bacterial infection in *Drosophila*. Thoracic injury is thought to lower bacterial resistance through activation of damage repair mechanisms and is thought to be independent of immune mechanisms (Chambers et al., 2014). We might therefore expect to see the same effect following thoracic pricking of virus. Orally infecting flies shows a range of sex-specific behavioural symptoms, with sub-lethal doses reducing daily locomotor activity in males after 3-6 days of infection (Gupta et al., 2017). Conversely, following oral infection with larger doses of DCV, females, but not males, have been shown to sleep more (Vale and Jardine, 2015c). These studies suggest we may not have seen an effect of DCV infection on activity, because infections were not severe enough to elicit behavioural symptoms or that any effect was concealed by a response to thoracic injury. Measuring the activity of flies later in infection might address these explanations, as this will enable flies to heal from thoracic injury and accrue a greater viral burden.

A potentially important nuance to how this variation in social aggregation might relate to disease transmission is that it was measured in groups of individuals with the same genetic background, sex and infection status. We controlled these

sources of variation within Petri dishes to pinpoint their influence over social aggregation and highlight their relative importance. Variation in these characteristics can produce population structure that potentially biases contact between individuals, and therefore transmission, in a variety of ways. Individuals with shared genotypes can be more likely to interact due predispositions to traits such as group size preference (Brown and Brown, 2000; Philippe et al., 2016) and aggression (Lea et al., 2010). Similarly, sexual interactions between males and females, as well as fighting and other forms of sexual competition, further alter contact networks within populations (Perkins et al., 2008; Silk et al., 2018). When present together, healthy hosts might also be able to avoid infected conspecifics by detecting the pathogen or cues of its pathology (Curtis, 2014). Future work aiming to characterise the influence of these sources of variation on heterogeneity in contact rate should consider how they change with, and are changed by, population structure.

The contrasting ways social aggregation and locomotor activity change following infection highlight the complexity of sources determining between-individual variation in disease transmission. This is complicated further by sex differences across and within these genetic backgrounds. The change induced by DCV infection on social aggregation but not locomotor activity also demonstrates the importance of considering multiple host behaviours. As locomotor activity did not change following infection in males or females across multiple genetic backgrounds, it could be a potentially useful predictor of transmission risk, as the locomotion of healthy hosts will approximate that of infected hosts. Similarly, healthy female social aggregation could be a reliable predictor of female social aggregation following infection as we detected no infection-induced behavioural change. Conversely, the distance between healthy males represents a baseline measure of aggregation, likely to be exceeded following infection. Importantly, while males move further apart when infected, some individuals still aggregate closer to one another than females of certain genetic backgrounds and may therefore present a greater transmission risk. This can be demonstrated by comparing males from the genetic background RAL-380 and females from RAL-75. Here, males aggregate

more closely when infected than females. Central to understanding the effect of this genetic and sex-specific variation in social aggregation and locomotor activity on heterogeneity in disease transmission is characterising their effect on contact rates. Additionally, future work should consider how these traits interact with other key determinants of transmission, such as infectiousness and infection duration, as these three components ultimately define disease transmission in conjunction with one another.

Chapter 4: Dissecting Genetic and Sex-Specific Host Heterogeneity in Pathogen Transmission Potential

I was assisted with the RNA extraction of viral load samples by Fergal H. Waldron.

I am the sole author of this text, with comments on earlier drafts from Pedro Vale.

Abstract

Heterogeneity in disease transmission is widespread and, when not accounted for, can produce unpredictable outbreaks of infectious disease. Despite this, precisely how different sources of variation in host traits drive heterogeneity in disease transmission is poorly understood. Here, we dissect the sources of variation in pathogen transmission using *Drosophila melanogaster* and *Drosophila* C Virus as a host-pathogen model system. We found that infected lifespan, viral growth, virus shedding, and viral load at death were all significantly influenced by fly genetic background, sex and female mating status. To understand how variation in each of these traits may generate heterogeneity in disease transmission, we estimated individual transmission potential by integrating data on virus shedding and lifespan alongside previously collected data on social aggregation. We found that ~15% of the variance in between-individual heterogeneity in disease transmission was explained by a significant interaction between genetic and sex-specific variation. We also characterise the amount of variation in viral load, virus shedding, and lifespan following infection that could be explained by genetic background and sex. Amongst the determinants of individual variation in disease transmission these sources of host variation play roles of varying importance, with genetic background generally playing the largest role. Our results highlight the

importance of characterising sources of variation in multiple host traits when studying disease transmission at the individual-level.

4.1 Introduction

Individual host heterogeneity in disease spread is commonly observed across a wide range of infectious diseases (Woolhouse et al., 1997; Lloyd-Smith et al., 2005; Paull et al., 2011). Such heterogeneity is so common that it has been generalised into the '20-80 rule' because of the frequent observation that 20% of hosts contribute to roughly 80% of transmission (Shaw and Dobson, 1995; Wilson et al., 2002; Woolhouse et al., 1997). More extreme forms of heterogeneity can result in very rare 'superspreading' individuals capable of causing large outbreaks of infectious disease in human and animal populations (Brooks-Pollock et al., 2014; Lloyd-Smith et al., 2005). A superspreader of particular infamy is Mary Mallon who became known as 'Typhoid Mary' by infecting over 50 people with *Salmonella typhi* while working as a cook in New York during the early 20th century (Marineli et al., 2013). More recently, the 2003 outbreaks of SARS in Singapore and Hong Kong were greatly accelerated by a few superspreading individuals who caused over 70% of all SARS transmission (Li et al., 2004).

Outbreaks of infectious disease are often difficult to predict, especially when the effect of superspreaders are not accounted for by traditional assessments of outbreak risk. A widely used metric for the rate of pathogen spread is the basic reproductive number, R_0, which estimates the average number of expected secondary infections caused by a single infected individual in a completely susceptible population. By focussing on the population average, R_0 conceals outliers with a potentially higher propensity to spread disease (Lloyd-Smith et al., 2005; Paull et al., 2012; VanderWaal and Ezenwa, 2016). A clearer understanding of what drives heterogeneity in disease transmission requires a framework capable of accounting for such between-individual variation, which could enable more efficient control strategies that specifically target and treat high-risk individuals (Lloyd-Smith et al., 2005). The importance of predicting high-risk individuals before outbreaks occur has pushed understanding the causes of heterogeneity in disease

transmission to the forefront of epidemiology and disease ecology research (Gervasi et al., 2015; Paull et al., 2012; Stein, 2011; VanderWaal and Ezenwa, 2016).

Despite being commonplace, the underlying causes of heterogeneity in pathogen transmission remain elusive. Individual variation in host contact networks may be an important factor: it was Typhoid Mary's position as a cook which exposed her to so many susceptible individuals. However, what enabled Typhoid Mary to stay in this role was her status as an asymptomatic carrier of the infection, which led to her release from quarantine on several occasions (Marineli et al., 2013). Similarly, the absence of symptoms in a number of SARS superspreaders delayed their admission to hospital and allowed them to continue spreading the virus (Centers for Disease Control and Prevention (CDC), 2003). These examples help demonstrate that achieving a detailed understanding of the sources of heterogeneity in pathogen transmission is challenging because it results from complex interactions between multiple host behavioural, physiological, and immune traits. By dissecting the underlying genetic and sex-specific sources of variation in these traits we can assess how they influence three key components of pathogen transmission: contact rate between infected and susceptible individuals, the likelihood that contact will result in infection, and the duration of infection (VanderWaal and Ezenwa, 2016).

Infected-susceptible host contact rate is predominantly determined by host behaviours affecting locomotion and aggregation. Contact rates are also affected by population density (Keeling and Rohani, 2007), social group size (Patterson and Ruckstuhl, 2013), and behavioural syndromes (Keiser et al., 2016). Social networks often exhibit extreme heterogeneity in the wild (Godfrey, 2013; Rushmore et al., 2013) and factors such as host genotype, sex condition, age and personality have been demonstrated to affect social aggregation in lab systems (de Bono and Bargmann, 1998; Keiser et al., 2016; Saltz, 2011; Siva-Jothy and Vale, 2019). Once individuals acquire an infection, their ability to clear and shed pathogens is chiefly determined by physiological and immune mechanisms. Variation in these mechanisms chiefly influence the likelihood of pathogen transmission and the

duration of infection (Grassly and Fraser, 2008; VanderWaal and Ezenwa, 2016). Many genetic and environmental sources of variation in physiological immunity have been described (Bou Sleiman et al., 2015; Lazzaro and Little, 2009; Ponton et al., 2013) including coinfection (Budischak et al., 2015; Lass Sandra et al., 2013), nutrition (Cornet et al., 2014; Vale et al., 2013), and stress (Beldomenico and Begon, 2010; Capitanio et al., 2008). It is relevant to note that most studies have addressed the effects of behavioural, physiological and immune traits on transmission in isolation of one another. However, there is increasing evidence that transmission heterogeneity is often explained by coupled heterogeneities in these traits and patterns of covariation (Bolzoni et al., 2007; Farrington et al., 2013; White et al., 2018). To fully understand the sources of heterogeneity in pathogen transmission, it is therefore essential to measure multiple behavioural, physiological, and immune traits in hosts.

In the present work we aimed to test how common sources of variation between individuals (genetic background, sex and mating status) contribute to individual heterogeneity in pathogen transmission potential. The fruit fly, *Drosophila melanogaster*, is a powerful and genetically tractable model of infection, immunity and behaviour (Apidianakis and Rahme, 2009; Sokolowski, 2001). This makes it an ideal model system to investigate heterogeneity in pathogen transmission in the highly controlled conditions of a laboratory. We infected males and females from a range of naturally derived genotypes with *Drosophila* C Virus (DCV), a horizontally transmitted fly pathogen that causes behavioural, physiological and metabolic pathologies (Arnold et al., 2013; Chtarbanova et al., 2014; Gupta et al., 2017; Vale and Jardine, 2015d). We then quantified host traits and infection outcomes that directly impact pathogen transmission: (1) the infected lifespan, (2) the internal viral load, (3) how much virus was shed, and (4) the viral load at death (VLAD). Finally, we integrated these measurements alongside previously described data on variation in social aggregation (Siva-Jothy and Vale, 2019) into a composite metric of individual transmission potential, V (Lloyd-Smith et al., 2005; VanderWaal and Ezenwa, 2016). Estimations of individual transmission potential, V, allowed us to

assess how genetic and sex-specific variation affects between-individual heterogeneity in pathogen transmission.

4.2 Materials & Methods

Flies & Rearing Conditions

Flies used in experiments were 3-5 days old and came from ten lines of the *Drosophila* Genetic Resource Panel (DGRP). These genetic backgrounds are five of the most resistant and susceptible to systemic *Drosophila* C Virus infection (Magwire et al., 2012). Virgin females were isolated from males within 7 hours of eclosion. Mated females and males were produced by rearing one female with one male for 24 hours. Mating was confirmed using oviposition within the following 24 hours and these egg's subsequent development. Flies were reared in plastic vials on a standard diet of Lewis medium at 18±1˚C with a 12 hour light:dark cycle. Stocks were tipped into new vials approximately every 14 days. One month before the experiments, flies were maintained at low density (~10 flies per vial) for two generations at 25±1˚C with a 12 hour light:dark cycle.

Virus Culture & Infection

The *Drosophila* C Virus (DCV) isolate used in this experiment was originally isolated in Charolles, France and grown in Schneider *Drosophila* Line 2 (DL2) as previously described (Vale and Jardine, 2015b), diluted ten-fold (10^8 infectious units per ml) in TRIS-HCl solution (pH=7.3), aliquoted and frozen at -70˚C until required. To infect with DCV, flies were pricked in the pleural suture with a 0.15mm diameter pin, bent at 90° ~0.5mm from the tip, dipped in DCV.

Measuring Lifespan and Viral Load at Death

Lifespan and viral load at death were measured in the same fly. Following DCV infection, flies were isolated and reared in standard vials. Flies were then monitored every day until all individuals died, whereupon they were removed from vials, fixed in 50µl of TRI-reagent and frozen at -70°C, to await DCV titre at death quantification.

For twenty-seven of thirty treatment groups, the lifespan following infection and viral load at death was measured for n=17-20, three treatment groups consisted of n=7-15 flies (Table S1).

Viral Load and Shedding Measurement Setup

Due to destructive sampling, we measured the viral load and shedding of single flies at a single time point, either 1-, 2- or 3-days post-infection (DPI). Following DCV infection, single flies were placed into 1.5ml Eppendorf tubes with ~50µl of Lewis medium in the bottom of the tube. To measure viral shedding, flies were transferred to tubes either immediately or 1 or 2 days after systemic infection for 24 hours. After living in these tubes for a further 24 hours, viral load samples were gathered by removing and homogenising flies in 50µl of TRI-reagent. Virus shedding samples were collected by washing tubes out with 50µl of TRI-reagent by vortexing. These samples were then frozen at -70°C, to await DCV quantification by qPCR. For each combination of sex and genetic background over the three days vial load and virus shedding was measured, n=7-15 flies were measured (Table S2-S4).

DCV RNA Extraction

RNA was extracted from viral load at death and viral shedding samples by Phenol-Chloroform extraction. Samples were thawed on ice for 30 minutes before being incubated at room temperature for 5 minutes to allow dissociation of nucleo-protein complex. Samples were then centrifuged at 12,000×g for 10 minutes at 4°C after which large debris was removed. For phase separation, samples were shaken vigorously for 15 seconds, 10µl of chloroform added, incubated at room temperature for a further 3 minutes before being centrifuged at 12,000×g for 15 minutes at 4°C. Following phase separation, the upper aqueous layer was removed from each sample and added to 25µl of Isopropanol, tubes were then inverted twice, before being centrifuged at 12,000×g for 10 minutes at 4°C. Precipitated RNA was then washed by removing the supernatant, and re-dissolving the RNA pellet in 50µl of 75% ethanol before being centrifuged at 7,500×g for 5 minutes at 4°C. RNA suspension was achieved by removing 40µl of the ethanol supernatant, allowing the

rest to dry by evaporation and dissolving the remaining RNA pellet in 20µl of RNase-free water. We extracted RNA from flies after 1, 2 or 3 days of infection using a semi-automatic MagMAX Express Particle Processor using the MagMAX-96 total RNA isolation kit manufacturer's protocol (Life Technologies, 2011) with the elution step extended to 18 minutes. RNA samples were stored at -70°C to await reverse transcription.

Reverse transcription & qPCR Protocol

Extracted RNA was reverse-transcribed with M-MLV reverse transcriptase and random hexamer primers, before being diluted 1:1 with nuclease free water. cDNA samples were stored at -20°C to await qPCR analysis. DCV titre was quantified by qPCR using Fast SYBR Green Master Mix in an Applied Biosystems StepOnePlus system. Samples were exposed to a PCR cycle of 95°C for 2 minutes followed by 40 cycles of: 95°C for 10 seconds followed by 60°C for 30 seconds. Forward and reverse primers used included 5'-AT rich flaps to improve fluorescence (DCV_Forward: 5' AATAAATCATAAGCCACTGTGATTGATACAACAGAC 3'; DCV Reverse: 5' AATAAATCATAAGAAGCACGATACTTCTTCCAAACC 3'). Across all plates, two technical replicates were carried out per sample. DCV titre was calculated by absolute quantification, using a standard curve created from a 10-fold serial dilution (1-10^{-12}) of DCV cDNA. Our detection threshold was calculated for each plate using the point at which two samples on our standard curve gave the same Ct value. The point of redundancy in a standard curve was taken to be equivalent to 0 viral particles. Due to our detection protocol measuring viral copies of RNA, we cannot comment on the viability of any detected virus. We transformed our measurements of viral RNA in order for them to represent the amount of virus growing inside a whole fly rather than the amount in the qPCR well sample. To account for dilution between RNA extraction and qPCR we transformed DCV RNA samples by a factor of 3125, to represent the amount of DCV growing in, or shed by, flies. The mean qPCR efficiency was 116% with a standard error of ±2.9%.

Calculating Between-Individual Variation in Transmission Potential, *V*

We used measures of virus shedding, lifespan following infection, and social aggregation to predict individual transmission potential. We integrated these measures based on a simple framework that describes transmission potential as a function of contact rate between susceptible and infected individuals, the likelihood that such contact will result in infection, and the duration of the infectious period (VanderWaal and Ezenwa, 2016). Using previously analysed data on social aggregation (Siva-Jothy and Vale, 2019; see Chapter 2), we used nearest neighbour distance as a measure of contact rate. Flies that aggregated more closely to conspecifics, have a higher contact rate, and are therefore more likely to spread DCV. We also assume that transmission likelihood increases with virus shedding. We therefore take the amount of virus shed by flies as a proximate measure of the likelihood that contact will result in infection. Using these traits, individual transmission potential, *V*, was calculated as:

$$V = \frac{(\text{Virus Shedding Titre}) * \text{Lifespan}}{(\text{Aggregation Distance})}$$

Aggregation distance, lifespan following infection and virus shedding were all measured in separate experiments. Therefore, to calculate V as a measure of individual transmission potential, we simulated theoretical individuals by bootstrapping trait values sampled from each of these three datasets. We simulated 60 individuals for each combination of sex and genetic background, assuming no specific covariance structure between traits, that is, all possible trait combinations were considered.

Statistical Analysis

Across all experiments, generalised linear models (GLMs) were used to analyse continuous response variables and logistic regressions were used to analyse proportions. An effect of sex or mating was analysed in separate models comparing males or virgin females to the same dataset of mated females, respectively.

To analyse lifespan, two GLMs were constructed containing a three-way interaction between genetic background, VLAD, and sex or mating (Table S6). The two GLMs for VLAD, contained either a two-way interaction between genetic background and sex or a two-way interaction between genetic background and mating (Table S6).

Due to zero-inflation, we used two models to sequentially analyse both viral load and virus shedding data. Viral load and virus shedding are broken down into qualitative (the proportion of non-zero values) and quantitative variation (differences between non-zero values). First, we conducted logistic regressions on all of the values in these datasets and analysed the proportion of values that were greater than zero. Logistic regressions analysing sex-differences in viral load included DPI (a 3-level factor: 1, 2 or 3 days) and an interaction between genetic background and sex (Table S6). For analysing the effect of mating in females on viral load, logistic regressions included DPI and an interaction between genetic background and mating (Table S6). Logistic regressions of virus shedding used a similar model that also included quantitative viral load as a predictor (Table S6). After these logistic regressions, zeroes were removed from all datasets to analyse the subset of positive-values. The GLMs used to analyse these subsets included the same predictors as their corresponding logistic regressions, for viral load: an interaction between genetic background and sex or mating, alongside DPI, with the inclusion of quantitative viral load for virus shedding (Table S6).

Due to zero-inflation V was also analysed with a logistic regression followed by a GLM. A logistic regression was used to analyse the proportion of V values that were greater than zero with a two-way interaction between sex and genetic background as predictors (Table S6). Zero-values of V were then removed from the dataset, and a GLM was used to analyse differences in the size of V, with an interaction between sex and genetic background included as a predictor (Table S6).

We calculated the amount of deviance and variance explained by predictors in logistic regressions and GLMs, respectively, by dividing the total deviance or variance explained by the model. Where appropriate, we corrected for multiple testing using Bonferroni correction. All statistical analyses and graphics produced in R 3.3.0 using the *ggplot2* (Wickham, 2016), *lme4* (Bates et al., 2015) and *multcomp* (Hothorn et al., 2008) packages.

4.3 Results

Lifespan Following Infection

Infected lifespan varied significantly between males and females and the extent of this variation differed between host genetic backgrounds (Figure 1a; Table 1). Genetic background explained the most variance of any predictor across models assessing mating (7%) and sex (10.9%). Interactions with sex and mating also explained a further 2.7% and 1.5%, respectively (Figure 5; Table 1). We found no evidence that mating affected the lifespan of females following DCV infection (Figure 1a; Table 1). Viral load at death (VLAD) was not affected by genetic background, sex or female mating status (Figure 1b; Table 2), and flies that died sooner following infection had greater VLAD than those that died later (Figure 1c; Table 1).

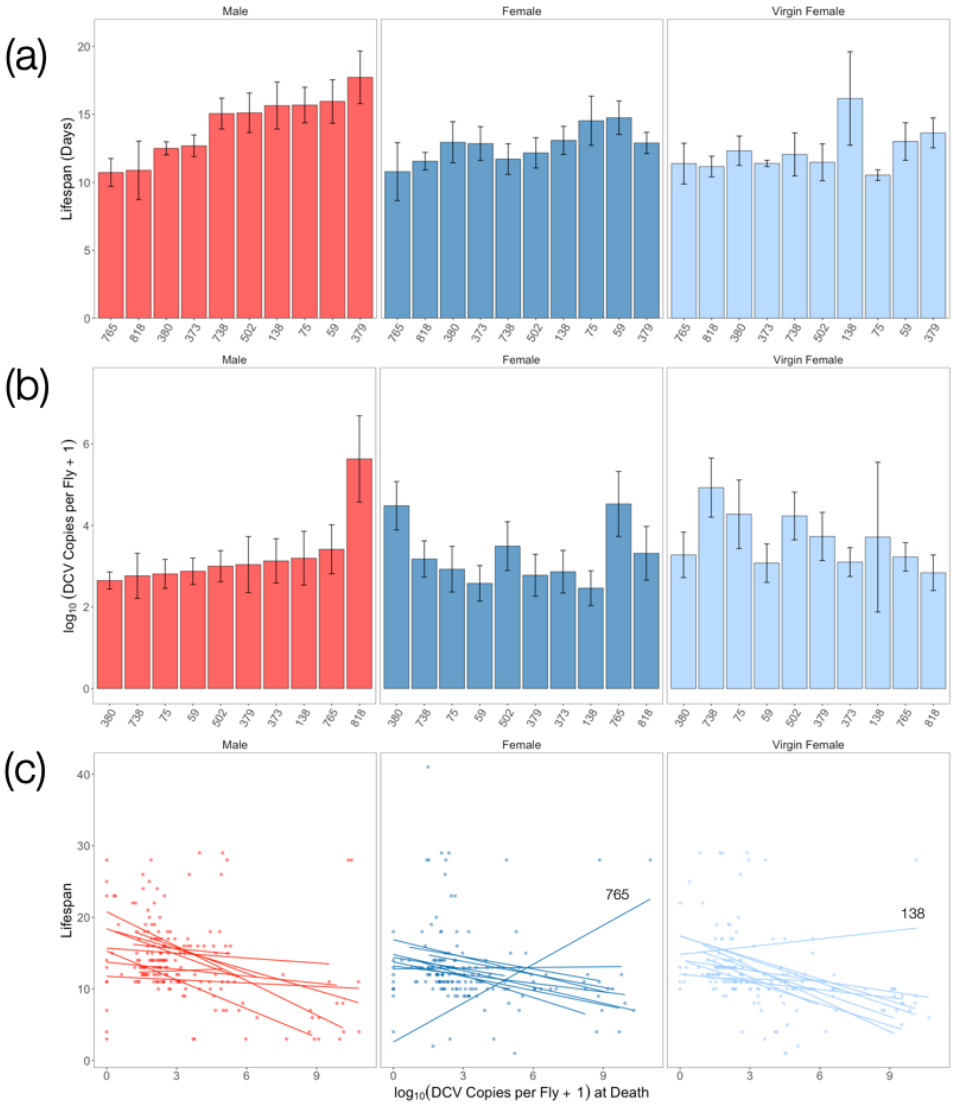


Figure 1. Mean±SE (a) lifespan in days following infection and (b) the viral load at death in males (red), mated females (blue), and virgin females (pale blue) of ten genetic backgrounds. The x-axis shows the line number form the DGRP panel and is in ascending order according to male flies. (c) the relationship between lifespan following infection and the viral load of flies at death. Each point is an individual male (red), mated female (blue), or virgin female (pale blue) fly. The nature of this relationship within each genetic background is represented by a line of best fit with outlier backgrounds labelled.

Response Variable	Predictor	Df	F	%Variance Explained	p-value
Lifespan Following Infection	Sex	1	2.00	0.6	0.16
	Genetic Background	9	3.92	10.9	<0.0001
	VLAD	1	38.9	12.1	<0.0001
	Sex*Genetic Background	9	0.96	2.7	0.47
	Sex*VLAD	1	5.46	1.7	0.02
	Genetic Background*VLAD	9	0.63	1.8	0.77
	Sex*Genetic Background*VLAD	9	2.67	7.4	0.005
	Mating	1	2.74	0.9	0.099
	Genetic Background	8	2.43	7.0	0.01
	VLAD	1	32.3	10.2	<0.0001
	Mating*Genetic Background	8	0.54	1.5	0.84
	Mating*VLAD	1	3.78	1.2	0.053
	Genetic Background*VLAD	8	1.71	4.9	0.087
	Mating*Genetic Background*VLAD	8	1.46	4.2	0.16

Table 1. Model outputs for the generalized linear modelling tests performed on lifespan following DCV infection. The VLAD acronym is used in place of 'viral load at death'. Separate analyses were used to test the effect of sex and mating in females.

Response Variable	Predictor	Df	F	% Variance Explained	p-value
Viral Load at Death (VLAD)	Sex	1	0.17	0.05	0.68
	Genetic Background	9	0.96	2.53	0.47
	Sex*Genetic Background	9	0.92	2.43	0.50
	Mating	1	1.90	0.57	0.17
	Genetic Background	8	1.30	3.5	0.24
	Mating*Genetic Background	8	0.93	2.49	0.50

Table 2. Model outputs for the generalized linear modelling tests performed on the viral load at death of flies infected with DCV. Separate analyses were used to test the effect of sex and mating in females.

Viral Load

A substantial number of flies did not have detectable loads of DCV. These zero-values reflect qualitative variation and are likely caused by viral titres below the detection threshold of our qPCR and therefore reflect individuals with very low DCV loads, or no virus at all. We found extensive genetic variation in qualitative DCV load (Figure 2a; Table 3) which was affected by sex (Figure 2a; Table 3) and female mating status (Figure 2a; Table 3). Relatively little deviance was explained by sex (0.002%), mating (0.13%), or genetic background in models testing sex (1.18%) and mating (2.83%) effects. The predictors that explained the most deviance were the interactions between genetic background and sex (5.58%) or mating (4.92%) (Figure 5; Table 3). The size of non-zero DCV loads reflects quantitative variation and was affected by similar interactions between mating and sex with genetic background (Figure 2b; Table 4). While <1% of variance was explained by sex and mating, much more was explained by genetic background (7.94% and 11%) alongside its interactions with sex (19.2%) and mating (4.38%; Figure 5; Table 4).

The number of detectable DCV loads decreased following 1-day post-infection (pairwise comparison, $p<0.0001$) and remained lower than day 1 at day 3 (pairwise comparison, $p=0.0016$). There were significant changes in quantitative DCV load variation over the first three days of infection. Viral load peaked 2-days post-infection (pairwise comparison, $p=0.0012$), before decreasing to the same level as 1-day post-infection at 3-days post-infection (pairwise comparison, $p=0.068$).

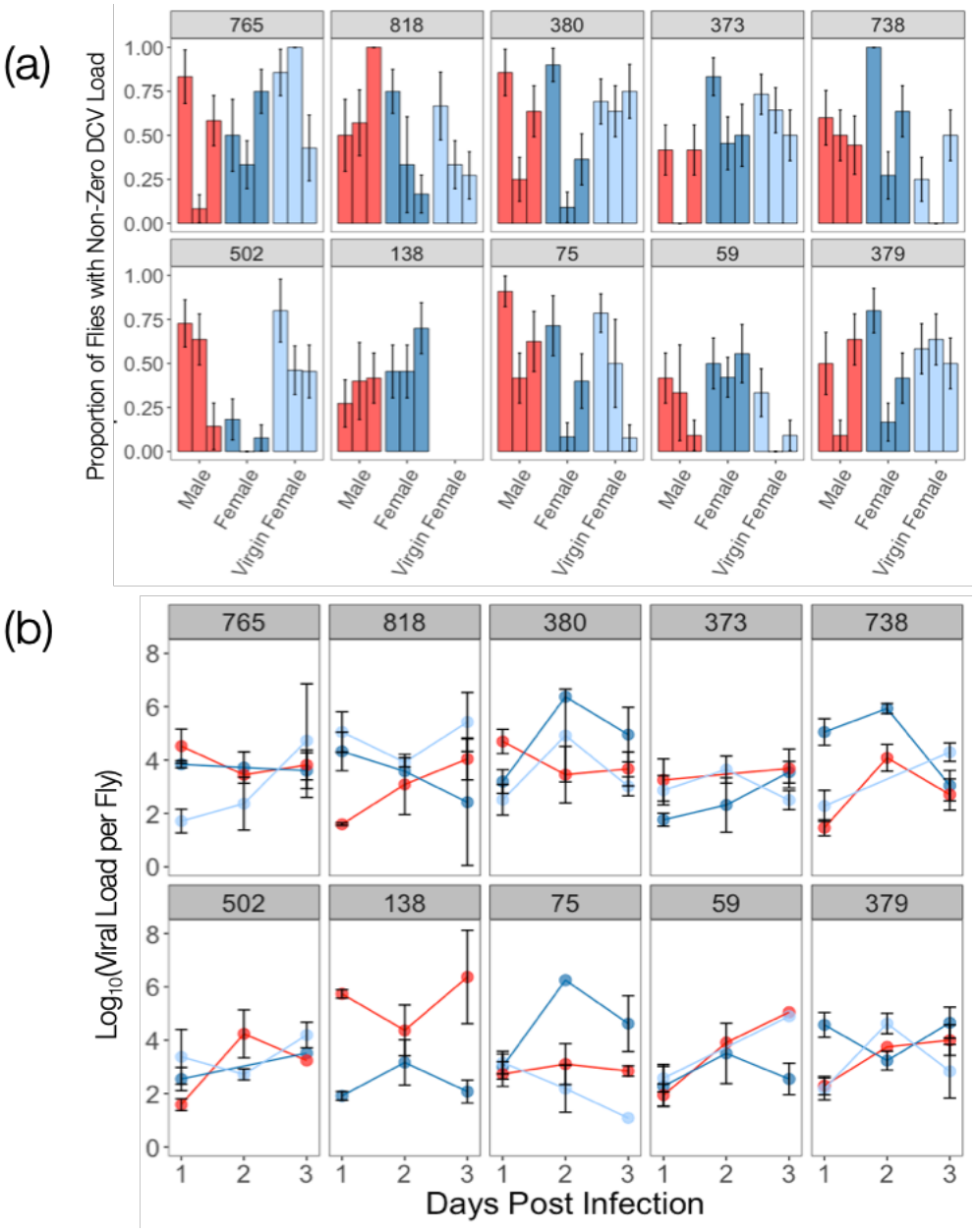


Figure 2. Mean±SE (a) proportion of flies with non-zero loads of DCV over the first 3 days of infection (day 1, 2 and 3 come first second and third, respectively) and the (b) viral titre of flies with non-zero DCV loads over the first 3 days of infection. Numbers in each panel denote the genetic background in the DGRP panel, while the colour of bars, points and lines represent sex and mating status. Males are shown in red, mated females in blue, and virgin females in pale blue.

Response Variable	Predictor	Df	X^2	% Deviance Explained	p-value
Qualitative DCV Load	Sex	1	0.019	0.002	0.89
	Genetic Background	9	9.58	1.18	0.39
	DPI	2	36.6	4.52	<0.0001
	Sex*Genetic Background	9	45.2	5.58	<0.0001
	Mating	1	1.01	0.13	0.31
	Genetic Background	8	22.4	2.83	0.008
	DPI	2	27.2	3.43	<0.0001
	Sex*Genetic Background	8	39	4.92	<0.0001

Table 3. Model outputs for the binomial logistic regression conducted on qualitative DCV loads (the proportion of non-zero DCV loads). The DPI acronym is used in place of 'days post-infection'. Separate analyses were used to test the effect of sex and mating in females.

Response Variable	Predictor	DF	F	% Variance Explained	p-value
Quantitative DCV Load	Sex	1	0.0062	0.003	0.94
	Genetic Background	9	2.24	7.94	0.02
	DPI	2	3.37	2.65	0.036
	Sex* Genetic Background	9	5.41	19.2	<0.0001
	Mating	1	0.68	0.26	0.41
	Genetic Background	8	3.18	11.0	0.0012
	DPI	2	4.66	3.60	0.01
	Mating* Genetic Background	8	1.42	4.38	0.19

Table 4. Model outputs for the GLM analysis conducted on quantitative DCV load (the titres of non-zero DCV loads). The DPI acronym is used in place of 'days post-infection'. Separate analyses were used to test the effect of sex and mating in females.

Virus Shedding

Similar to measures of viral load, we did not detect DCV in the shedding of a number of flies. Here, we interpret zeroes to be reflective of individuals that shed very low titres of DCV, or no virus at all. Qualitative variation in DCV shedding was significantly affected by genetic background, with sex modulating the extent of this difference (Figure 3a; Table 5). Sex however, explained <1% of the deviance, while genetic background and its interaction with sex explained 2.2% and 3.07% (Figure 5). Mating did not affect qualitative DCV shedding (Figure 3a; Table 5) and explained <1% of the deviance (Figure 5; Table 5). In flies where DCV was detected in shedding, quantitative DCV shedding was affected by genetic background and the extent of this variation was determined by female mating status, but not sex (Figure 3b; Table 6). The amount of variance explained by sex and in our models was <1%, in comparison with genetic background (9.48% and 5.82%) and its interactions with sex (8.87%) or mating (6.53%) (Figure 5; Table 6). Qualitative and quantitative DCV shedding peaked at day 2 (Figures 3a; Tables 5 & 6, pairwise comparisons, $p<0.0001$). Across all treatment groups, there was no significant relationship between viral load and shedding (Figure S1; Table 6).

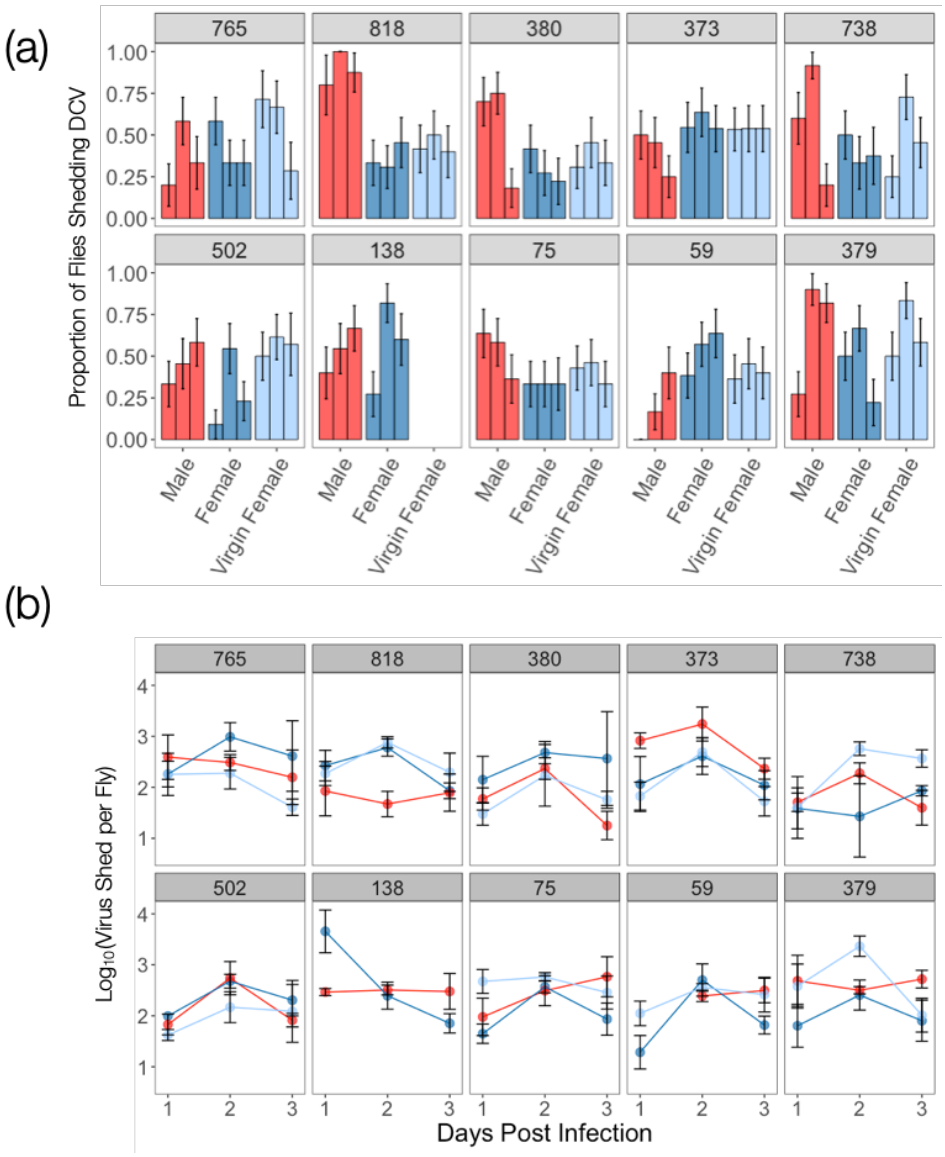


Figure 3. Mean±SE (a) proportion of flies shedding non-zero titres of DCV over the first 3 days of infection (day 1, 2 and 3 come first second and third, respectively) and the (b) titre of the non-zero virus shedding subset over the first 3 days of infection. Panels denote genetic background, while the colour of bars, points and lines represent sex and mating status. Males are shown in red, mated females in blue, and virgin females in pale blue.

Response Variable	Predictor	Df	χ^2	% Deviance Explained	p-value
Qualitative DCV Shedding	Sex	1	4.93	0.64	0.026
	Genetic Background	9	17.6	2.27	0.04
	Viral Load	1	0.03	0.004	0.85
	DPI	2	25.1	3.25	<0.0001
	Sex*Genetic Background	9	23.8	3.07	0.005
	Mating	1	1.33	0.18	0.25
	Genetic Background	8	19.0	2.53	0.025
	Viral Load	1	1.10	0.15	0.29
	DPI	2	7.66	1.02	0.022
	Mating*Genetic Background	8	8.12	1.08	0.42

Table 5. Model outputs for the GLM analysis conducted on qualitative DCV shedding (the proportion of sheddings with non-zero readings of DCV). The DPI acronym is used in place of 'days post-infection'. Separate analyses were used to test the effect of sex and mating in females.

Response Variable	Predictor	Df	F	% Variance Explained	p-value
Quantitative DCV Shedding	Sex	1	0.67	0.28	0.42
	Genetic Background	9	2.52	9.48	0.009
	Viral Load	1	5.03	4.21	0.007
	DPI	2	0.23	0.095	0.63
	Sex*Genetic Background	9	1.73	6.53	0.082
	Mating	1	0.22	0.098	0.64
	Genetic Background	8	1.44	5.82	0.17
	Viral Load	1	11.2	10.1	<0.0001
	DPI	2	0.18	0.08	0.67
	Mating*Genetic Background	8	2.46	8.87	0.014

Table 6. Model outputs for the GLM analysis conducted on quantitative DCV shedding (the subset of shedding with non-zero readings of DCV). The DPI acronym is used in place of 'days post-infection'. Separate analyses were used to test the effect of sex and mating in females.

Variation in Transmission Potential, *V*

We incorporated the lifespan and virus shedding data described above alongside previously gathered data on genetic and sex-specific variation in social aggregation to calculate individual disease transmission potential, *V* (Lloyd-Smith et al., 2005; VanderWaal and Ezenwa, 2016). As a result of many flies not shedding DCV (Figure 3a), the distribution of transmission potential, *V*, was zero-inflated (Figure 4a). Zero values of *V* represent individuals with no transmission risk (Figure 4a), as flies that shed no virus had no transmission potential, irrespective of their aggregation and lifespan. The distribution of *V* was also characterised by a right-extreme tail, comprised of individuals with high-risk transmission potentials relative to the population average (Figure 4a). Qualitative variation in *V* (the proportion of flies where $V>0$) differed between males and females with the extent of this difference also determined by genetic background (Figure 4b; Table 7). Sex (0.28%), genetic background (2.3%) and the interaction between the two (2.83%) explained relatively little deviance in our models (Figure 5; Table 7). In quantitative variation in *V*, sex explained <1%, while genetic background and its interaction with sex explained 4.13% and 11.4% of variance respectively (Figure 5; Table 8).

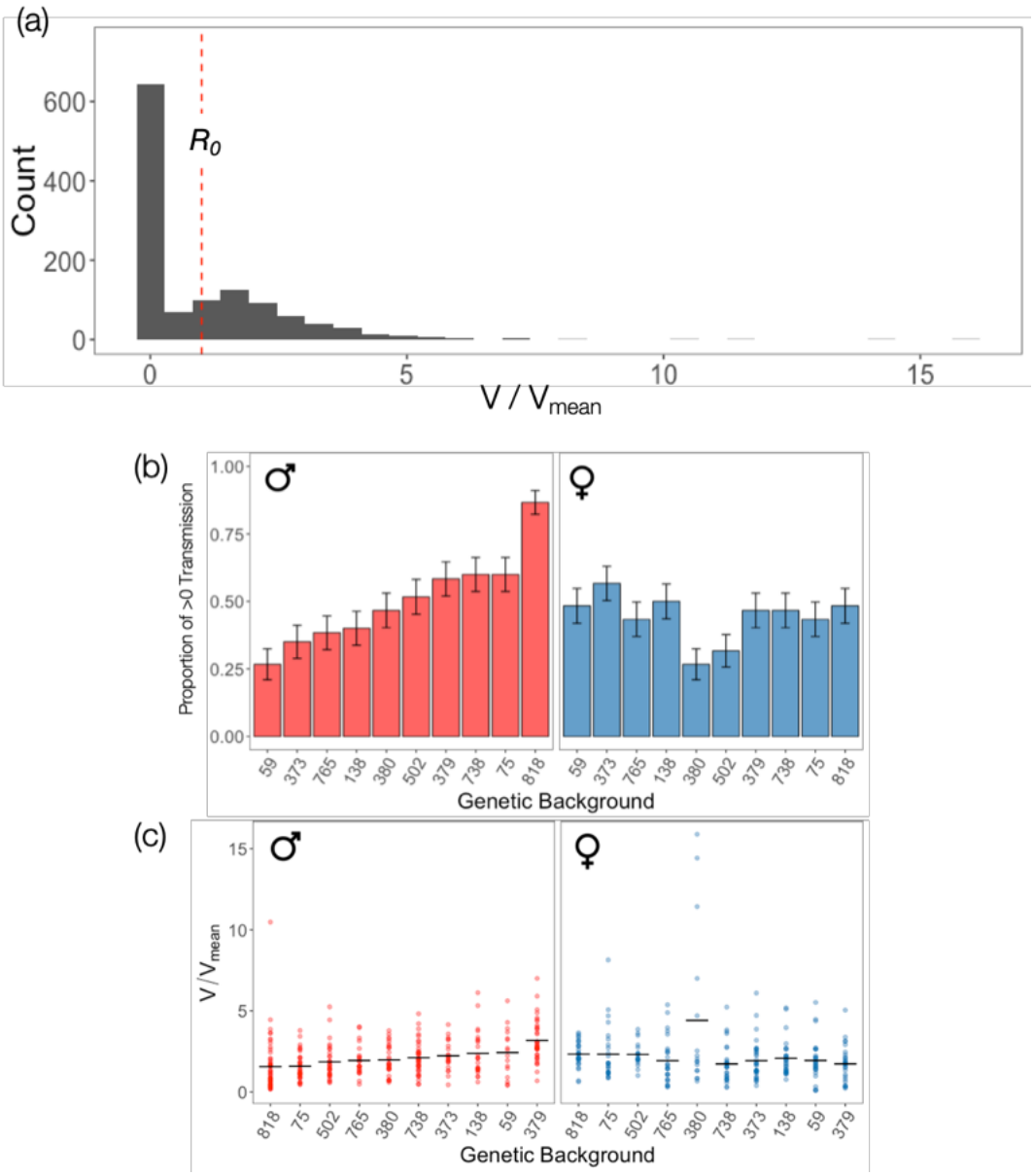


Figure 4. (a) the population-level distribution of transmission potential (*V*) relative to the mean of the population. The red dashed line demarcates the average transmission potential of the population (similar to R_0), a traditional metric used to describe a population's outbreak risk. The mean±SE of (b) the proportion of flies with a non-zero transmission potential and (c) the transmission potential of flies with a non-zero transmission potential. In figure panels (b) and (c) sex denoted and marked by colour with males in red and females in blue. The x-axis of panels (b) and (c) is in ascending order of the male genetic backgrounds.

Response Variable	Predictor	Df	χ^2	% Deviance Explained	p-value
Qualitative *V*	Sex	1	4.58	0.28	0.032
	Line	9	38.2	2.30	<0.0001
	Sex*Line	9	47.0	2.83	<0.0001

Table 7. Model outputs for the logistic regression analysis conducted on qualitative *V* (the proportion of non-zero *V* values).

Response Variable	Predictor	Df	F	% Variance Explained	p-value
Quantitative *V*	Sex	1	0.077	0.01	0.78
	Line	9	2.51	4.13	0.008
	Sex*Line	9	6.94	11.4	<0.0001

Table 8. Model outputs for the GLM analysis conducted on quantitative *V* (the subset with non-zero *V* values).

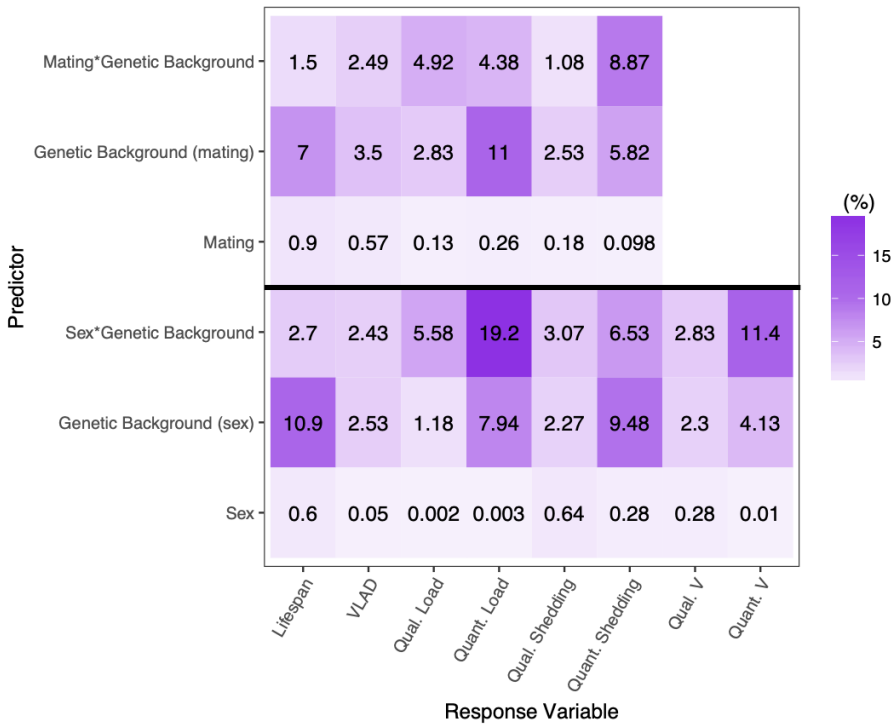


Figure 5. Summary of the percentage of variance or deviance explained by a subset of predictors in analyses of disease transmission potential and outcomes of infection. Predictors involving mating (top 3 rows) and sex (bottom 3 rows) refer to the variance and deviance explained by models comparing mated females with virgin females and mated females with mated males, respectively (Table S6).

4.4 Discussion

We identified genetic and sex-specific variation in three key outcomes of DCV infection: lifespan following infection, virus shedding, and virus load. When combined with social aggregation data, this variation resulted in genetic and sex-specific variation in individual transmission potential, *V*. While all of these outcomes of infection influence transmission potential, due to many individuals not shedding any virus, virus shedding exerted more influence over *V* than variation in lifespan following infection and social aggregation. Due to this central role, below we discuss potential explanations for the effect of mating, as well as genetic and sex-specific variation on virus shedding, and link these to genetic and sex-specific variation in *V*.

4.4.1 The effect of host genetic background in generating heterogeneity in transmission

The genetic variation in virus shedding affected both qualitative and quantitative variation in DCV shedding. As the distributions of neither social aggregation nor lifespan following infection were zero-inflated, variation in virus shedding appears to be a key determinant of qualitative and quantitative variation in *V*. Differences between genetic backgrounds in qualitative shedding was a key determinant of variation in *V*, as there is no risk of pathogen transmission in the absence of shedding. Among individuals that shed DCV, between-individual heterogeneity in *V* was achieved through different routes. Some genetic backgrounds, such as males from RAL-818, showed a high proportion of individuals that are likely to spread DCV (Figure 4b), but only to relatively few individuals (Figure 4c). Conversely, other groups, such as females of the RAL-380 genetic backgrounds, showed one of the lowest proportions of individuals able to achieve transmission (Figure 4b), but the individuals that did achieve transmission include outliers with values of *V* that were orders of magnitude higher than the population average (Figure 4c).

Quantitative and qualitative variation in DCV shedding differed in how they were affected by host genetic background. Qualitative variation was affected by genetic background as part of an interaction with host sex, while this interaction has no significant effect on quantitative DCV shedding (Tables 5 & 6). Similar differences are seen in the amount of deviance and variance genetic background explains in models of qualitative and quantitative variation in DCV shedding. Genetic background accounts for only 2.27% of deviance in qualitative DCV shedding whereas it accounts for 9.48% of the variance in quantitative DCV shedding (Figure 5). Genetic variation therefore appears to play an important role in determining shedding and affects qualitative and quantitative shedding in different ways. Similar effects of genetic backgrounds on parasite shedding have been reported in the Ramshorn snail species, *Biomphlamaria glabrata*, during infection with *Schistosoma mansoni.* Genetic backgrounds differ in how many parasite eggs they shed and how quickly they start shedding after infection (Tavalire et al., 2016). The differences we see in the proportion of flies shedding DCV may be caused by a similar pattern of variation in individual's delaying virus shedding. Delaying the onset of shedding could be affected by a range of DCV infection symptoms. These include paralysis of muscles in the crop organ of the foregut, abdominal swelling, broad nutritional stress and reduced defecation rate (Chtarbanova et al., 2014).

Genetic background also appears to play a key role in transmission potential, we detected a significant effect on both qualitative and quantitative variation in *V.* The amount of deviance and variance explained by genetic background does not hugely differ (2.3% and 4.13%, respectively). However, when part of an interaction with sex, genetic background accounts for 11.4% of the variance in quantitative variation in DCV shedding, whereas this same interaction only accounts for 2.83% of the deviance in qualitative variation in shedding (Figure 5). Alongside other studies, this highlights the potential significance of genetic variation in pathogen shedding to generating transmission heterogeneity. For example, genetic variation in transmission was demonstrated using families of turbot fish (*Scophthalmus maximus*) which produced outbreaks that differed in how quickly individuals

showed symptoms of infection and died (Anacleto et al., 2019). Shedding may underlie this genetic variation in transmission as it was not directly measured and there were no significant differences in infection duration and contact rate (Anacleto et al., 2019). Common garden experiments have revealed shedding dynamics capable of influencing the population-level transmission dynamics of wild populations of the plant, *Plantago lanceolata*. In controlled laboratory settings, multi-strain coinfection was shown to increase the number of spores released of the fungal pathogen, *Podosphaera plantaginis*. Measures of natural populations have also demonstrated outbreak severity increases at higher levels of coinfection (Susi et al., 2015a). The relationship between spore shedding and coinfection has also been shown to be affected by host genotype, with genotypes significantly differing in the number of spores released over a number of days post-infection (Susi et al., 2015b). Genetic variation in transmission potential has also been demonstrated in the freshwater ciliate, *Paramecium caudatum*, following *Holospora undulata* infection (Fellous et al., 2012). The genotype of the first individual to be infected was a key determinant of pathogen transmission as host genotype appears to affect the infectious potential of shed pathogens (Fellous et al., 2012). *H. undulata* infectiousness increases with host population density, as reduced variation in contact rate makes infectiousness the primary determinant of transmission (Magalon et al., 2010).

4.4.2 The effect of host sex in generating heterogeneity in transmission

We also observed clear qualitative and quantitative differences in *V* between males and females, which is suggestive of sex-specific variation in disease transmission. While the extent of any difference between males and females is also determined by genetic background, a greater proportion of males tend to transmit DCV than females across these backgrounds. In DCV shedding, a greater proportion of males from several genetic backgrounds (RAL-379, RAL-738 and RAL-818) shed DCV than females (Figure 3a). Interestingly, we see significant sex-specific differences in qualitative, but not quantitative, variation in DCV shedding. Other work has also shown a number of sex differences in pathogen and parasite shedding (Sanchez et

al., 2011; Sheridan et al., 2000; Thompson et al., 2017). Often these biases link to differences in the selection pressures applied by sexual reproduction (Duneau and Ebert, 2012). Comparisons of mated and virgin female flies revealed mating effects which produced quantitative, but not qualitative, differences in shedding. While we did not measure *V* in virgin females, this mating effect may offer explanations for the sex differences seen in shedding and therefore *V*.

Sex-specific variation in qualitative differences in shedding exerts a significant influence over shedding (Figure 3a). It is important to note however, that in isolation, sex accounts for a miniscule 0.64% of the deviance in qualitative variation in shedding. Sex appears to play a more important role in conjunction with genetic background, the interaction between the two explaining 3.07% of deviance (Figure 5). While significant, sex-specific variation may play a relatively minor role in shedding. A variety of factors appear to underlie sex-differences in shedding across host-pathogen systems. For example, male-biased infection is common to many mammal hosts but generally absent from arthropod hosts (Sheridan et al., 2000). In the water flea, *Daphnia magna*, parasite spores are released into the environment upon death and females have been shown to release significantly more than males (Thompson et al., 2017). In the vole, *Microtus gryalis*, the faeces of females contains significantly more parasite eggs than that of males (Sanchez et al., 2011). Given that we see female-biased mortality to DCV infection (Figure 1a), it is perhaps surprising that shedding is not also female-biased. This could be due to shedding being measured during the first three days of infection, whereas mortality occurred much later. We might therefore see sex-differences in shedding during the later stages of infection.

Both the qualitative and quantitative differences in *V* between males and females were determined alongside genetic background. While sex explained very little deviance and variance in qualitative and quantitative variation in *V* (Figure 6), its interaction with genetic background explained 2.83% and 11.4 %, respectively. Sex could therefore be an important source of variation in individual disease

transmission. Sex differences in transmission or virus shedding, lifespan and social aggregation are commonly observed in a wide range of species (Duneau and Ebert, 2012; Ferrari et al., 2004; Kaltz and Shykoff, 2001; Sanchez et al., 2011). Sex-specific variation has been relatively well-studied because sexes are easily distinguished in the wild, and examples of sexual dimorphism in physiological and behavioural traits are relatively common (Duneau and Ebert, 2012). Many mammalian hosts exhibit male-biased transmission (Ezenwa et al., 2016; Grear et al., 2012; Luong et al., 2009; Rhines, 2013), although there are exceptions of female-bias (Sanchez et al., 2011). In the white-footed mouse, *Peromyscus leucopus*, male-biased transmission is thought to be driven by sex differences in contact network connectivity, which has been linked to testosterone production (Foo et al., 2017; Grear et al., 2012). Testosterone may be particularly relevant to transmission as its immunosuppressive (Foo et al., 2017) effects may also alter infectiousness and infection duration.

4.4.3 Female Mating Status in Shedding

Mated and virgin females did not qualitatively differ in DCV shedding; however, individuals did exhibit quantitative variation in shedding. While a negligible amount of the variance in quantitative shedding was explained by mating, the interaction between mating and genetic background explained 8.87% of the variance (Figure 5). This suggests that alongside host genetic background, mating might exert an important level of influence over shedding. One potential explanation for this mating effect are post-mating physiological changes in the intestine that can increase defecation rates (Apger-McGlaughon and Wolfner, 2013). However, if this change is responsible for the significant effect of female mating, the virgin females from particular genetic backgrounds that shed more than mated females (Figure 3b) may be tolerant to these physiological changes. Relatively few have considered how mating affects aspects of disease transmission outside of contact rates (Altizer et al., 2003; Thrall et al., 2000). Particularly alongside other work in *Drosophila* that has demonstrated female-specific costs of infection (Kubiak and Tinsley, 2017; Short et

al., 2012), this result highlights the importance of mating-induced physiological changes to transmission heterogeneity.

The difference between qualitative and quantitative variation in shedding relates to assumptions regarding how often DCV is shed. If DCV is always present in shedding, measures of zero reflect quantities of virus that are below the detection threshold of qPCR. While this could result in infectious individuals evading detection, as oral infection typically requires very high dosage (Gupta et al., 2017; Palmer et al., 2018), low-titre zero-values pose a smaller transmission risk. If DCV is not always shed, within-individual variation in when shedding occurs could be central to transmission heterogeneity (Chen et al., 2013). This is particularly relevant to our study as virus shedding was only measured at a single time point per fly, and we do not know how shedding, and therefore *V*, may change over time. Within-host, temporal variation in shedding is observed in a range of host-pathogen systems (Chen et al., 2013; Matthews et al., 2006; Mideo et al., 2008). For example, avian hosts tend to shed more parasites during the late afternoon (Brawner III and Hill, 1999; Martinaud et al., 2009).

By combining measures of virus shedding, lifespan and social aggregation into a simple framework our work demonstrates that genetic and sex-specific variation can affect individual heterogeneity in disease transmission potential. We also show that genetic and sex-specific variation, as well as mating, can produce variation outcomes of infection. Alongside its interaction with sex, genetic background explains 5.41% of qualitative, and 15.54% of quantitative, individual variation in transmission potential. While our results do not implicate a particular genetic background, males generally present a greater transmission risk than females. In addition to highlighting high-risk individuals, our results are congruous with the observation that the majority of infected individuals produce very few, if any, secondary cases of infection. Non-infectious individuals are particularly relevant to predicting outbreaks of infectious disease as they obscure high-risk individuals in traditional, population-wide estimations of outbreak risk. Our findings demonstrate

the benefit of using a model laboratory system as well established as *D. melanogaster* to study disease transmission. The number of available protocols and methodologies are central to considering multiple traits central to disease transmission and holistically studying their underlying determinants.

Chapter 5: Population-Level Disease Dynamics Reflect Individual Heterogeneities in Transmission

Lauren White and I collaboratively designed the simulation experiments described below. The simulations and random forest analysis were performed by Lauren White, who also provided an initial draft of their associated methods sections.

I am the sole author of this text, with comments on earlier drafts from Meggan Craft, Lauren White and Pedro Vale.

Abstract

Host heterogeneity in disease transmission is widespread and presents a major hurdle to predicting and minimizing pathogen spread. Using the *Drosophila melanogaster* model system infected with *Drosophila* C virus, we integrate empirical measurements of individual host heterogeneity in social aggregation, virus shedding, and disease-induced mortality into an epidemiological framework that simulates outbreaks of infectious disease within theoretical populations. We use these simulations to calculate individual variation in disease transmission and apportion this variation to specific components of transmission: social network degree distribution, infectiousness, and infection duration. The empirically-observed variation produces substantial differences in individual transmission potential, providing evidence for genetic and sex-specific effects on disease dynamics at a population level. Manipulating variation in social network connectivity, infectiousness, and infection duration in simulated populations reveals these components affect disease transmission in clear and distinct ways. We report the implications of this genetic and sex-specific variation in disease transmission and discuss implications for appropriate control methods given the relative contributions made by social aggregation, virus shedding, and infection duration to transmission in other host-pathogen systems.

5.1 Introduction

Individual heterogeneity in host traits affecting disease transmission has major consequences for the predictability and severity of outbreaks of infectious disease, and in extreme cases can lead to 'superspreaders' or 'supershedders' of infection (Craft, 2015; Gopinath et al., 2014; Lloyd-Smith et al., 2005; White et al., 2017). An individual's transmission potential can be described as a function of its contact with susceptible individuals, the likelihood of that contact resulting in infection, and the length of time that individual remains infectious (Anderson and May, 1981; VanderWaal and Ezenwa, 2016). While the underlying causes of heterogeneity in transmission are poorly understood, each of these components may be affected by genetic variation in pathogen traits, the behavioural and physiological traits of the host, and their interaction with environmental factors (Hawley and Altizer, 2011; Lopes et al., 2016; Susi et al., 2015b; Vale et al., 2013). While the effects of host contact behaviour on heterogeneity in pathogen transmission have been widely investigated (Keiser et al., 2017; Lloyd-Smith et al., 2004; May and Anderson, 1987), the role of variation in host physiological traits in generating heterogenous pathogen transmission are less clearly understood (VanderWaal and Ezenwa, 2016; White et al., 2018). Moreover, the relative roles of these traits, and how they interact with one another in natural systems remain difficult to isolate and quantify.

One commonly used descriptor of how likely a disease is to spread through a population is the basic reproductive number, R_0, which denotes the mean number of secondary cases caused by an infected individual in a susceptible population (Anderson and May, 1981; Elderd et al., 2013). R_0 is one the most widely used metrics in epidemiology, commonly used to predict outbreaks of infectious disease and as a theoretical tool to model pathogen evolution (Gandon et al., 2016). However, a potential shortcoming of R_0 is that it reflects population averages, making it a poor predictor of disease outbreaks that arise due to extreme individual heterogeneity in transmission (Cross et al., 2007; James O Lloyd-Smith et al., 2005; Lloyd-Smith et al., 2006). One way to address this shortcoming is to move beyond

the population average transmission reflected in R_0, and measure the R_0 equivalent for each individual in that population, termed V (James O Lloyd-Smith et al., 2005; Lloyd-Smith et al., 2006; VanderWaal and Ezenwa, 2016). This approach has the advantage of explicitly measuring the distribution of individual transmission (where the mean of the distribution is R_0) and can be useful in identifying individuals at the most extreme of this distribution which could be likely superspreaders of infection. Another advantage of applying the basic reproduction number to individuals is that it provides an experimentally tractable framework to partition the variance in individual transmission among a range of behavioural, physiological and immune phenotypes that may lead to variation in V (VanderWaal and Ezenwa, 2016)

Notwithstanding these advantages, quantifying the behavioural, physiological and immune traits underlying the number of infections produced by a single individual remains tremendously challenging, particularly in wild or natural disease settings. One potentially useful approach is to measure V and its components by experimentally infecting model systems under controlled laboratory settings in order to quantify the roles of physiological and behavioural host heterogeneity on pathogen transmission (Keiser et al., 2016; Lopes et al., 2016; Susi et al., 2015a; Vale et al., 2013). This experimental approach offers the advantage of minimising environmental variation and allowing highly replicated measurements of individual host traits. However, such studies may be limited in their ability to extrapolate the effects of measured at the level of individual hosts to population-level epidemic dynamics. Mathematical modelling/in silico experiments are a useful tool to efficiently test different hypotheses in larger/scaled up populations and infer patterns across scales (Lloyd-Smith et al., 2009), but many theoretical studies often rely on assumptions about the extent of heterogeneity in host traits, in the absence of adequate empirical information (McCallum et al., 2017; VanderWaal and Ezenwa, 2016). The ideal approach is therefore to use mathematical modelling of epidemiological dynamics where as many parameters as possible are informed by experimental data measured on individual hosts in controlled laboratory settings.

Here we use this approach combining experiment and theory to test how population-level disease transmission dynamics are affected by empirically measured levels of variation in pathogen shedding, lifespan following infection and social aggregation. We previously measured individual-level variation in behavioural and physiological traits that are relevant to pathogen transmission in the fruit fly (*Drosophila melanogaster*) when infected with its viral pathogen *Drosophila* C Virus (DCV; see chapters 3 & 4). These experiments leveraged genetic and sex-specific sources of variation in three traits that likely affect DCV transmission which occurs via larval or adult feeding (Keebaugh and Schlenke, 2014): the degree of group-level social aggregation (as an indicator of potential contact rate); mortality rate (which defines the duration of infection); and how much DCV each individual sheds into their environment (as a proxy measure of infectiousness).

We address three questions about the interactions of different types of behavioural and physiological heterogeneity in pathogen transmission. First, we asked if genetic and sex-specific variation in social aggregation, virus shedding, and duration of infection – as measured in lab setting – would result in different predicted epidemics in theoretical populations. In this initial set of simulations, theoretical populations were comprised of individuals with traits that were representative of the phenotypic heterogeneity in males or females of a single genetic background. By simulating and comparing epidemics in host populations comprised of a single sex and one genetic background, we focused on genetic and sex-specific sources of variation in disease transmission.

Second, to test the relative importance of the genetic background and sex of the index case vs. group composition and how variation in transmission potential is affected by the diversity of the susceptible population, we simulated epidemics in populations where individuals' traits are sampled from a larger phenotypic distribution, including males and females from ten genetic backgrounds. In these simulations, we varied the genetic background and sex of the index case.

Third, to test the relative importance of variation in specific host traits on epidemic dynamics, we compared epidemic dynamics of populations exhibiting empirically-measured levels of variation in social aggregation, viral shedding and mortality, to populations where we constrained variation in these traits to the population mean.

5.2 Materials & Methods

Measuring social aggregation, viral shedding and infection duration in infected *D. melanogaster*

Simulations were parameterised using experimental data on host aggregation, mortality, and viral shedding described in Chapters 3 and 4. Readers are directed to that publication for a detailed description of data collection. Briefly, we established systemic infections with DCV in ten lines (Table 1) from the *Drosophila* Genetic Resource Panel (DGRP) (Mackay et al., 2012), chosen because they are extremes of susceptibility to DCV systemic infection (Magwire et al., 2012). DCV was cultured and prepared as described previously (Siva-Jothy et al., 2018a). Systemic infections were achieved by pricking flies in the thorax near the pleural suture with a 0.5mm entomological needle dipped in DCV (Merkling and van Rij, 2015). To measure lifespan following DCV infection, single flies were monitored daily until dead. DCV shedding was measured 1, 2, and 3 days after infection in 1.5ml Eppendorf tubes. Flies were removed from tubes after 24 hours and processed for qRT-PCR. DCV shed into the tube was collected by adding 100µl of TRI-reagent and thoroughly vortexing. TRI-reagent was removed for RNA extraction and reverse transcription before being quantified by qPCR (Siva-Jothy et al., 2018a). To measure social aggregation, photos were taken of groups of 10-12 flies of the same genetic background, sex and infection status, in 55mm petri dishes. The mean number of neighbours each individual in a Petri dish had within circles of a 10mm, 15mm and 20mm threshold radius was calculated using the coordinates of each fly generated with the ImageJ multipoint tool.

Simulation Methods

We used these empirical measurements from *D. melanogaster* to develop an individual-based, stochastic, static network model that tests how the sex-specific and genetic variation in viral shedding, susceptibility and social aggregation translates to differences in disease dynamics. Using a susceptible-infected (SI) model we simulated DCV transmission in a closed population with no births and where infected individuals can die (Anderson and May, 1992). Alongside empirically measured traits, we also tested the effect of pathogen viability and the relative infectiousness of supershedders. The effects of all parameters on outbreak dynamics were tested in a fully-factorial design. For each parameter set, 500 simulations were conducted for a population of 1000 individuals over the course of 1000 time steps (Tables 1, 2 & 3). Key metrics to measure outbreak dynamics included: fadeout likelihood, maximum number of infected individuals, outbreak duration, and time to maximum number of infected individuals. Code to conduct these simulations was written in R (Version 3.4.4) and is available at: https://github.com/whit1951/Drosophila

Social network degree distribution. To generate a simulated contact network reflecting contact rates of different phenotypes, we used a proxy for social aggregation: the number of neighbours within a set threshold radius. Individuals (nodes) within the prescribed threshold radius share an edge where transmission is possible. Using the number of neighbours within this radius for each fly, we derived a functional degree distribution for our simulated populations of interest. From this empirical degree distribution, we sampled 1000 times based on the number of individuals needed for the simulated network. This produced a network where the number of connections between nodes (mean degree rather than network density) was maintained between empirical and simulated populations. The dynamics of faecal-oral DCV transmission are poorly understood (Huszar and Imler, 2008; Webster et al., 2015), but the virus is seen to readily proliferate through laboratory stocks of *Drosophila* (Kapun et al., 2010). To account for this and assess the relative importance of possible direct transmission routes, we consider the number of

neighbours within 10, 15 or 20mm of one another and derive simulated social networks from these distinct degree distributions. Transmission is only possible between infected (I) and susceptible (S) flies within this set infectious distance. Importantly, using social aggregation as a proximate measure of contact rate assumes the likelihood of contact with DCV is proportional to an individual's proximity to an infected fly.

Infectiousness

We estimated infectiousness (κ_j) for any given infected individual, j, from our empirical measurements of viral shedding. The untransformed distribution of this data is highly skewed and zero-inflated, with some rare flies shedding exceedingly high viral titres (i.e., so-called supershedders), and others not shedding any virus at all (within the technical limit of detection). To account for this disparity, we used the natural log to transform our viral load shed distribution and divided these values by the greatest amount of virus shed, constraining infectiousness values between 0 and 1.

The amount of virus needed to ensure transmission is unclear. To account for this, we considered a 'scaled infectiousness' (η) parameter which had two levels, 1 or 2. This parameter reflects two hypotheses: (1) only supershedders at the upper end of our shedding distribution ensure 100% transmission, with all other individuals having a probability less than one; or (2) average and non-zero shedders could still shed enough to ensure infection, but supershedders increase the likelihood of transmission relative to average counterparts. The two levels of scaled infectiousness, 1 and 2, were implemented by multiplying our measure of infectiousness (κ_j) by 1 or 2 respectively.

Another factor that may affect transmission is the viability of DCV in the environment without a host. To account for this, we included a transmission efficiency (τ) parameter into our model. The three levels, τ=0.1, 0.5, or 1, altered infectiousness by multiplying the infectiousness value by 0.1, 0.5, and 1 respectively. The levels of

transmission efficiency correspond to 10, 50, and 100% probability of transmission. Both scaled infectiousness (η) and transmission efficiency (τ) were held constant in simulations unless specifically mentioned.

Infection duration

DCV results in death for infected flies, making our empirical measurement of the time between inoculation and death an ideal measure of infection duration (μ). We calculated mortality rate as the inverse of empirical disease-related mortality for a given population. Once infected, individuals experienced a weighted coin flip probability of dying [$\beta(1, 1/\mu)$] at each time step.

Transmission rate

Combining all the elements above, transmission rate β_{ij} between a susceptible individual (i) and infectious host (j) is subject to the infectiousness of the infectious host (κ_j), the scaled infectiousness (η=1 or 2), the transmission efficiency of the pathogen (τ=0.1, 0.5 or 1), and whether or not an edge exists in the network between individuals i and j $\left(x_{ij} = \begin{Bmatrix}1\\0\end{Bmatrix}\right)$:

$$\beta_{ij} = \kappa_j \eta \tau x_{ij}$$

With individual-specific disease related mortality (μ_j), transmission rate translates to differences in the number of susceptible (S) and infected (I) individuals at each time step according to:

$$S_{t+1} = -\sum \beta_{ij} S_i I_j$$

$$I_{t+1} = \sum \beta_{ij} S_i I_j - \sum \mu_j I_j$$

For each individual at each time step, infection and mortality were stochastic processes governed by Bernoulli draws, e.g., $Bernoulli(\beta_{ij})$ and $Bernoulli(\mu_j)$.

Theoretical simulation #1

The effect of genetic and sex-specific variation in social aggregation, viral shedding and susceptibility on pathogen transmission potential. We scaled-up empirical degree distributions for males and females of our ten genetic backgrounds to a theoretical population size of 1000. In each simulated population, flies were of the same sex and genetic background. We allowed infectiousness, duration of infection, and social aggregation to vary based on empirical measurements for each combination of sex and genetic background. For each individual simulation, we generated a new network from the scaled-up degree distribution, and randomly selected an individual from the network to start as the index case.

Parameter	Levels
Genetic background	RAL-59, RAL-75, RAL-138, RAL-373, RAL-379, RAL-380, RAL-502, RAL-738, RAL-765, RAL-818
Sex	Female, Male
Threshold radius (r)	10mm, 15mm, 20mm
Pathogen transmission efficiency (τ)	0.1, 0.5, 1
Scaled infectiousness (η)	1, 2

Table 1. Factorial design and specifications for simulations testing the effect of genetic and sex-specific variation in social aggregation, viral shedding and susceptibility on population-level disease dynamics. We conducted 500 replicates per parameter set with 1000 individuals in the network. Simulations were allowed to run for 1000-time steps.

Theoretical simulation #2

The effect of population diversity on pathogen transmission potential. Many natural host populations have highly variable levels of diversity which can significantly affect

host-pathogen dynamics (Ostfeld and Keesing, 2012). To test if differences in transmission potential are robust to the diversity of the index case, we simulated populations where males and females of all ten genetic backgrounds were combined in equal proportion. More specifically, the simulated, scaled-up populations of 1000 individuals were comprised of 20 sub-populations each containing 50 sampled individuals drawn from the larger experimental distribution for each respective line/sex combo. Individuals maintained their respective empirical distributions for aggregation, infectiousness, and duration of infection according to their genetic background and sex combination. These simulated populations therefore reflect a relatively diverse population. We then varied which genetic background and sex combination served as the index case with 500 replicates per index case phenotype (Table 2).

Parameter	Levels
Index genetic background	RAL-59, RAL-75, RAL-138, RAL-373, RAL-379, RAL-380, RAL-502, RAL-738, RAL-765, RAL-818
Index sex	Female, Male
Threshold radius (r)	10mm, 15mm, 20mm
Pathogen transmission efficiency (τ)	0.1, 0.5, 1
Scaled infectiousness (η)	1, 2

Table 2. Factorial design and specifications for simulations testing the effect of susceptible host diversity on disease transmission potential. We conducted 500 replicates per parameter set on networks of 1000 individuals (20 subgroups of 50 individuals each representing each sex/line combo). Simulations ran for 1000-time steps.

Theoretical simulation #3

The consequences of variation in social aggregation, viral shedding and disease-related mortality on disease dynamics. To determine the relative importance of empirical variation in social aggregation, viral shedding, and disease-related mortality on disease transmission in a heterogeneous population, we simulated

populations derived from the variation seen across all genetic backgrounds and both sexes. To determine the effect of population-level variation, we systematically constrained the variation in all three host traits to the population's mean, individually and alongside one another. During these simulations, the unconstrained traits were free to vary according to the empirical measurements (Table 3). In the case of degree of the network, we rounded this value to ensure a whole number, which is essential for contact network formation (e.g. an individual cannot have 2.5 contacts). For example, to look at the effect of social aggregation by itself, we allowed social aggregation to take on the degree distribution of the entire heterogenous population, but constrained viral shedding and infection duration to the empirically-measured means across all genetic backgrounds and both sexes.

Parameter	Levels
Threshold radius (r)	10mm, 15mm, 20mm
Pathogen transmission efficiency (τ)	0.1, 0.5, 1
Scaled infectiousness (η)	1, 2
Number of nodes in simulated network	1000
Vary social aggregation	TRUE, FALSE
Vary infectiousness	TRUE, FALSE
Vary infection duration	TRUE, FALSE

Table 3. Experimental design for Experiment 3. We conducted 500 replicates per parameter set. Simulations ran for 1000-time steps.

Random Forest Analysis

Parsing out the effects of individual variables in simulation modelling can be challenging because of collinear effects and sensitivity of frequentist measures of significance to sample size (White et al., 2014). Random forest analysis is a machine

learning approach that readily handles non-linear relationships between variables (Cutler et al., 2007). Here we utilize the *cforest* function from the *party* package in *R* to look at variable importance scores, which reflect the relative influence of each variable in the prediction of the random forest model (Breiman, 2001; Strobl et al., 2009). The *party* package, in particular, addresses some of the potential biases of the original *randomForest* package that may result from continuous variables or variables with more categories (Strobl et al., 2009). For each theoretical experiment, we generated 1000 trees – at this level, no changes in variable importance order resulted from changes in the random seed suggesting a robust ranking order (Strobl et al., 2009). We reported variable importance scores as mean decrease in accuracy (a measure of permutation importance rather than node impurity), which describes the loss in accuracy resulting from randomly permuting the given variable (Strobl et al., 2009, 2007).

Outbreak Descriptors

We used five metrics to measure and characterise simulated outbreaks of infectious disease: fadeout likelihood, basic reproductive number (R_0), maximum number of infected individuals, the time taken to reach the maximum number of infected individuals, and outbreak duration. Fadeout likelihood represents the probability of an outbreak not occurring following the infection of the index case. It is the proportion of index cases that fail to transmit infection to at least one susceptible individual before dying from infection. We use R_0 as a measure of the number of secondary cases of infection caused by the index case for the duration of the simulation.

5.3 Results

As described in the Methods, in theoretical experiments 1 and 2, we tested full-factorial combinations of genetic background, sex, threshold radius, transmission efficiency and scaled infectiousness. In experiment 3, we tested full-factorial combinations of variation in infectiousness, social aggregation and infection

duration, threshold radius, transmission efficiency and scaled infectiousness. Here, we present results with a threshold radius of 15mm, a transmission efficiency of 1, and a scaled infectiousness of 2 (Figure 1). We focussed on this combination of parameters to promote pathogen transmission in our simulations, while also allowing us to subsequently test the importance of direct transmission by comparing the 15mm threshold radius to 10mm and 20mm.

Our findings were robust to changes in the values of various combinations of sex, genetic background and variation in infectiousness, social aggregation and/or infection duration, alongside transmission efficiency, scaled infectiousness and threshold radius. Summary figures describing the fadeout likelihood, basic reproductive number (R_0), maximum number of infected individuals, the time taken to reach the maximum number of infected individuals, and outbreak duration, for every parameter combination are available in the Chapter 5 Appendices (Figures S1-12).

5.3.1 Theoretical experiment #1

Individual variation in host infectiousness, social aggregation, and infection duration produced variation in population-level, pathogen transmission dynamics.

The variation in empirical treatment groups produced distinct outbreaks of infectious disease in populations comprised solely of one genetic background and sex (Figures 1-2). Random forest analysis suggested that the two top predictors for outbreak likelihood were genetic and sex-specific variation (Figure 3a). Given a successful outbreak, host genetic and sex-specific variation also affected the maximum number of infected individuals at any given time step (Figures 2b & 3b) and outbreak duration (Figures 2c & 3c). However, host genetic background and sex were less important than the threshold radius used to derive social network degree distribution for both outcomes (Figure 3b & 3c) and less important than transmission efficiency for predicting the maximum number of infected individuals (Figure 3b).

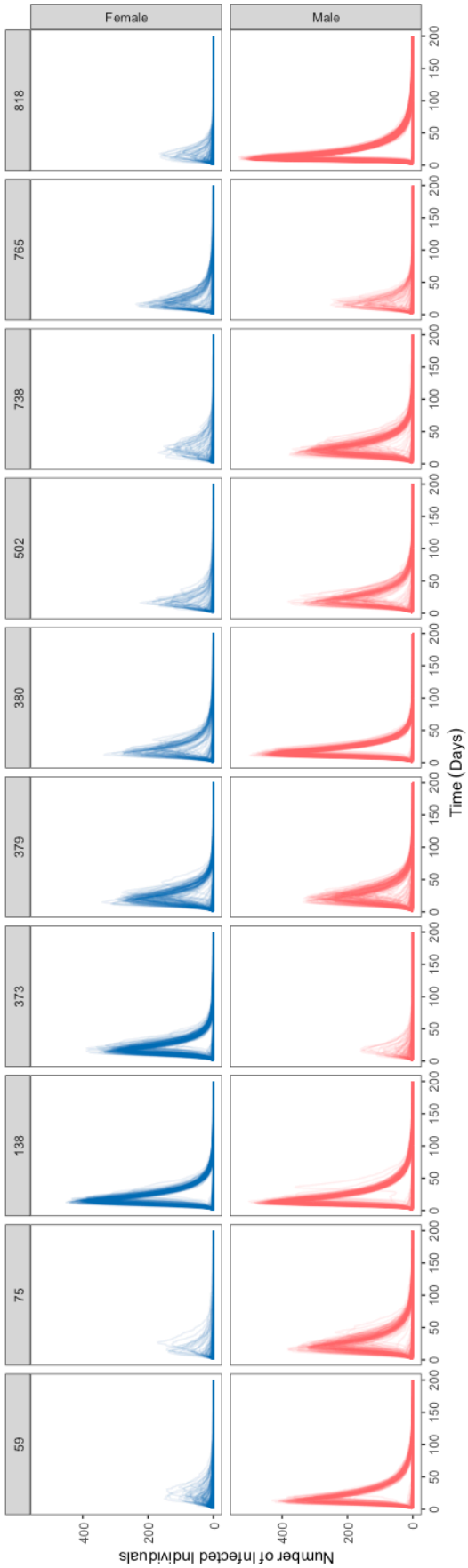


Figure 1. Simulation time courses of populations comprised of either male (red) or female (blue) individuals of the same and genetic background (columns) for simulation experiment #1. Across all of these simulations, parameters outside of host genetic background and sex are fixed; threshold radius (r)= 15mm, transmission efficiency (τ)=1 and scaled infectiousness (η)=2.

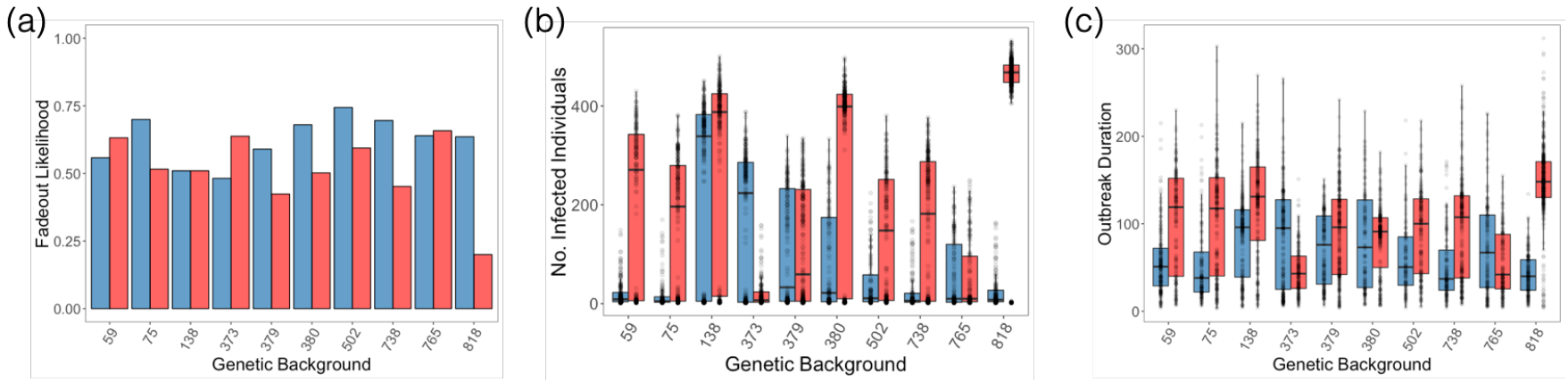


Figure 2. Summary statistics of time course simulations of populations comprised of either male (red) or female (blue) individuals of the same genetic background (x-axis). Across all of these simulations, parameters outside of host genetic background and sex are fixed; threshold radius (r)= 15mm, transmission efficiency (τ)=1 and scaled infectiousness (η)=2. All simulations were included to measure: (a) the proportion of simulations that resulted in fadeout; and, in the subset of simulations where fadeout did not occur, and disease spread from the index case; (b) the maximum number of infected individuals at any given time step; and (c) the number of time steps infected by the index case.

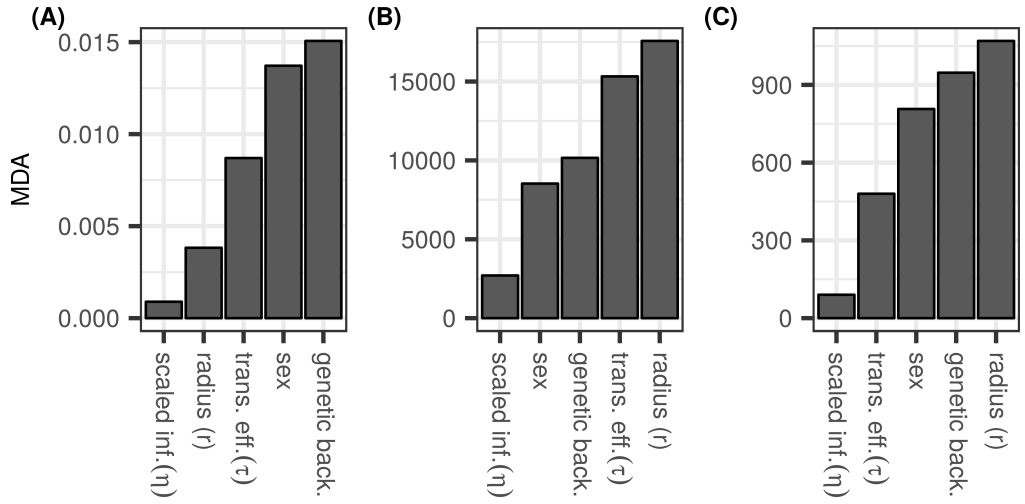


Figure 3. Results of variable importance analysis for theoretical experiment #1 with n=1000 trees using cforest function from the *party* package in *R*. Simulation variables are listed on x-axis. Y-axis describes variable importance (mean decrease in accuracy [MDA]). Which variables most determine: (A) whether the infection spread beyond initially infected individual? If so, which factors determine: (B) how many individuals infection reaches? and (C) how long it lasts?

5.3.2 Theoretical experiment #2

Effects of the index case outweighed by heterogeneity in the susceptible population.

The genetic background or sex of the index case did not alter outbreak dynamics in diverse populations where 20 empirical treatment groups were equally sampled to create a heterogeneous population (Figure 4a & 5). This was true for all outbreak descriptors (Figure 4 & 5). With no variation across empirical treatment groups, the importance of threshold radius, transmission efficiency, and scaled infectiousness influenced outbreaks, but in a consistent and predictable manner. Values conducive to greater infectiousness produced more likely and larger outbreaks (Figure 4a & 5). Based on the random forest analysis, threshold radius and transmission efficiency were the top two predictors for fadeout likelihood, maximum number of infected individuals (Figure 5), outbreak duration, and R_0(Figure S13).

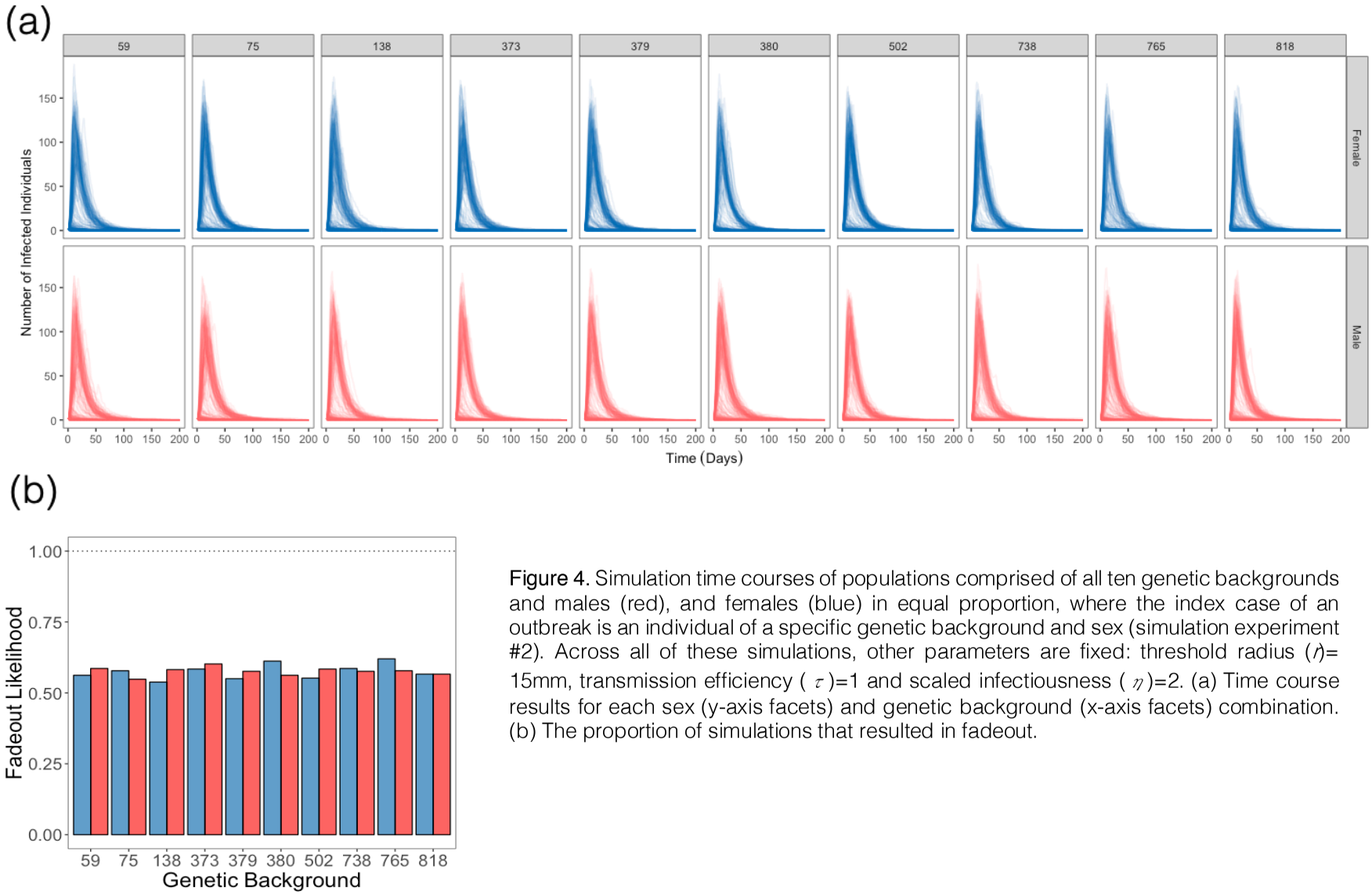


Figure 4. Simulation time courses of populations comprised of all ten genetic backgrounds and males (red), and females (blue) in equal proportion, where the index case of an outbreak is an individual of a specific genetic background and sex (simulation experiment #2). Across all of these simulations, other parameters are fixed: threshold radius (r)= 15mm, transmission efficiency (τ)=1 and scaled infectiousness (η)=2. (a) Time course results for each sex (y-axis facets) and genetic background (x-axis facets) combination. (b) The proportion of simulations that resulted in fadeout.

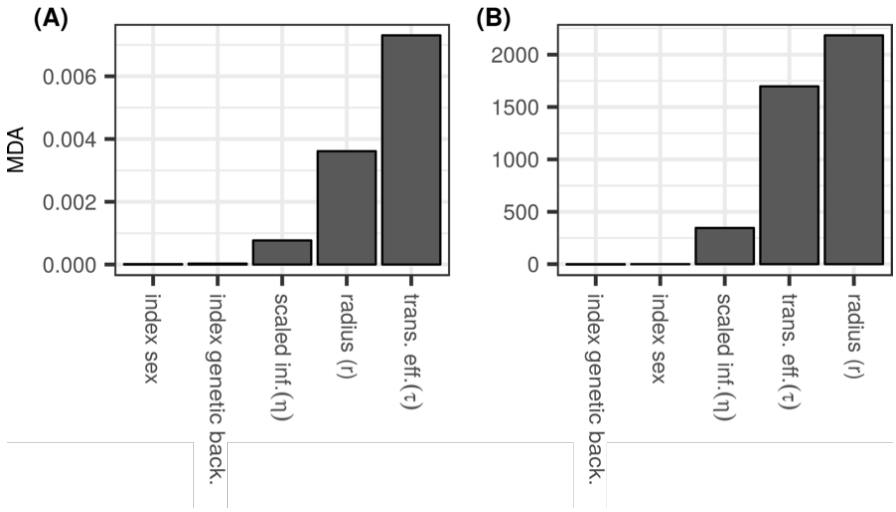


Figure 5. Results of variable importance analysis for Experiment #2 with n=1000 trees using cforest function from the *party* package in *R*. Variables are listed on x-axis. Y-axis describes variable importance (mean decrease in accuracy [MDA]). Which variables most determine: (A) whether the infection spread beyond initially infected individual? If so, which factors determine (B) how many individuals became infected?

5.3.3 Theoretical experiment #3

Variation in infectiousness made outbreaks less likely to occur, spread to fewer individuals, and persist in the population for longer.

Constraining the infectiousness of a population to the mean (0.23, 0.46 for scaled infectiousness (η) levels 1 and 2, respectively) of the empirical distribution increased the outbreak severity. This is clearly seen in outbreak time courses (Figure 6), making outbreaks more likely (Figure 7a), infect more individuals (Figure 6 & 7b), and persist in the population for longer (Figure 6 & 7c). The only parameter that was not positively affected by constraining infectiousness to the mean was the time taken to reach the maximum number of infected individuals (Figure 7c). Here, limiting variation in infectiousness made outbreaks more predictable, reducing the variance of the time taken to reach the maximum number of infected individuals (Figure 7c). According to the random forest analysis, variation in infectiousness was the top predictor for whether or not an outbreak spread beyond the initially infected individual (Figure 8a).

Variation in social aggregation makes outbreaks more severe. It does not, however, influence outbreak likelihood.

When social network degree distribution of simulated populations was confined to the mean of the empirical data (2, 3 and 4 for threshold radii of 10, 15 and 20mm respectively), outbreaks became less severe (Figure 6). Simulated DCV spread to fewer individuals (Figure 7b), at a slower rate (Figure 7c) and was quicker to die-out than in simulations where all three transmission components varied freely (Figure 7d).

Variation in disease-related mortality did not affect epidemic outcomes.

When constrained to the mean of the empirical data (13.6 days), we found disease-related mortality had little to no effect on any aspect of disease outbreak (Figure 6). Constraining variation in disease-related mortality did not alter outbreak likelihood or severity (Figures 6 & 7a-c). This is supported by the random forest analysis which identified infection duration as the least important predictor across outbreak metrics (Figure 8a-c).

Variation in infectiousness, followed by social aggregation, is the most influential component of transmission.

An increase in the maximum number of infected individuals is only seen when variation in infectiousness is constrained. Interestingly the same effect is seen in simulations where other traits are constrained alongside virus shedding, despite this differing substantially from the effects of social aggregation and infection duration when constrained alone (Figure 6-8). A similar, overruling effect is seen when social aggregation and infection duration are constrained simultaneously, and virus shedding varies freely; outbreak dynamics are similar to the cases where only aggregation is constrained (Figure 6-8).

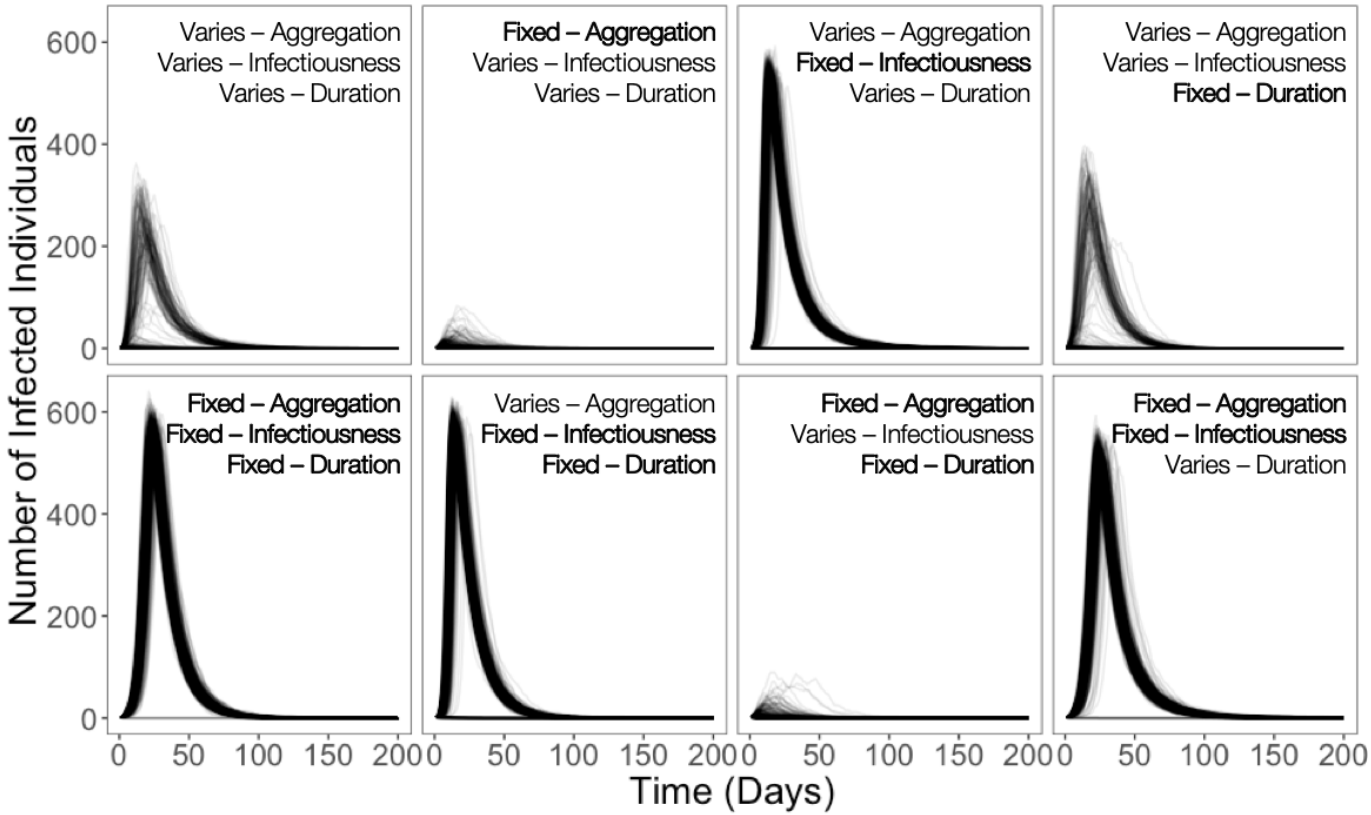


Figure 6. Simulation time courses of populations where aggregation, infectiousness and duration variation are derived from the breadth of the entire population- s variation rather than for a single genetic line and sex combination (simulation experiment #3). In each panel, the variation of a particular set of components is confined to the population- s mean. Across all of these simulations, parameters outside of host genetic background and sex are fixed: threshold radius = 15mm, transmission efficiency =1 and scaled infectiousness =2.

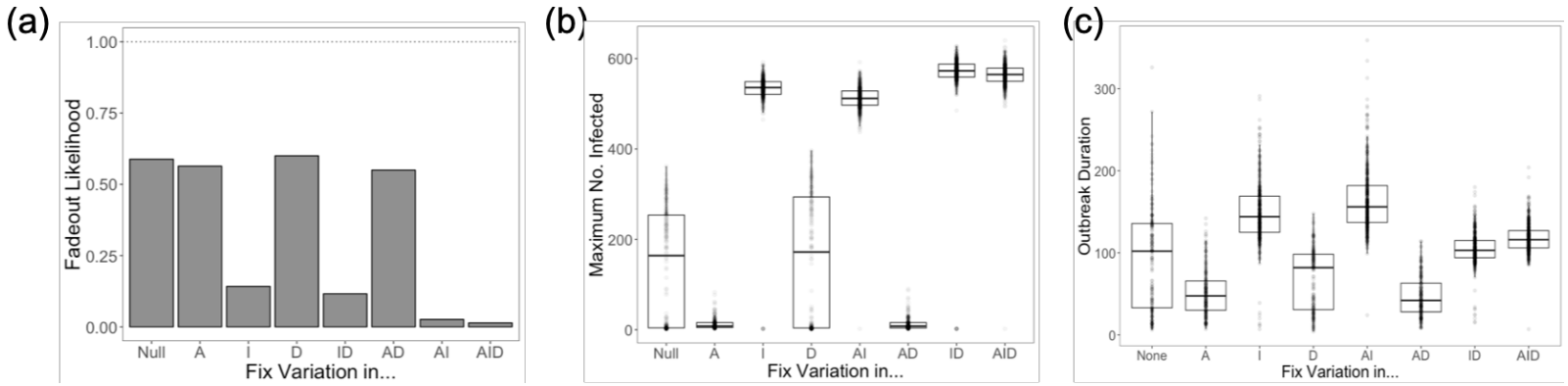


Figure 7. Summary statistics of time course simulations where individual variation is determined by the variation seen across all genetic backgrounds and sexes (simulation experiment #3). Across all of these simulations, threshold radius = 15mm, transmission efficiency =1, and scaled infectiousness =2. The x-axis of all panels sees variation in aggregation (A), infectiousness (I) and infection duration (D), and all their combinations fixed to the population mean. All simulations were included to measure: (a) the proportion of simulations that resulted in fadeout; (b) the maximum number of individuals infected during the simulation; (c) the time until maximum prevalence was reached.

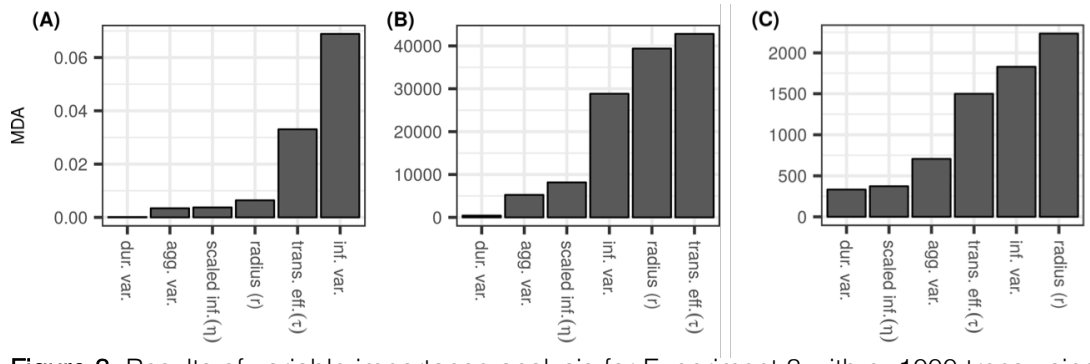


Figure 8. Results of variable importance analysis for Experiment 3 with n=1000 trees using cforest function from the *party* package in *R*. Variables are listed on x-axis. Y-axis describes variable importance (mean decrease in accuracy [MDA]). Which variables most determine: (A) whether the infection spread beyond initially infected individual? If so, which factors determine (B) how many individuals become infected and (C) how long it lasts.

Increasing the threshold radius increased outbreak severity but not likelihood.

Manipulating the distance transmission can occur over, across all three theoretical experiments, made outbreaks more severe (Figure 9), but was not as strong a predictor of outbreak likelihood (Figure 3, 5 & 8). Furthermore, we can see a non-linear relationship in the threshold radius when variation in social aggregation, virus shedding, and disease-related mortality were constrained to the population mean. When simulations derived social network according to one of three definitions of contact (10, 15 and 20mm), we see stark differences between 10 and 15mm, when compared to 15 and 20mm, indicating an important threshold value for the distance social interactions are drawn over. This distance was the most important predictor of outbreak parameters across almost all simulations (Figure 3, 5 & 8).

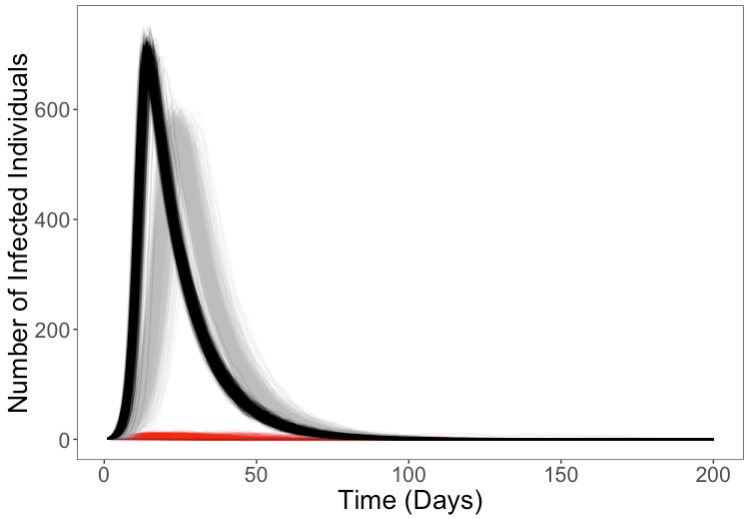


Figure 9. Simulation time courses of populations where aggregation, infectiousness and duration variation are fixed to the mean of the empirical population's variation independently of genetic background and sex. A total of 3 radii were used to derive population social networks from empirically measured data, 10mm (red), 15mm (grey) and 20mm (black). Parameters outside of this infectious radius were constrained to one value; transmission efficiency =1 and scaled infectiousness =2.

5.4 Discussion

We found substantial between-individual differences in disease transmission, constituting genetic and sex-specific variation in transmission potential. Crucially, in relatively homogenous populations comprised of single sex and genotype combinations, heterogeneity in the index case produced major differences in population-level outbreak dynamics, including making outbreaks more likely, broader reaching, and longer lasting. Despite the size of some of these differences however, variation in the index case's transmission potential exerted little influence over population-level outbreak dynamics in diverse host populations. We also found that population-level variation in social aggregation, virus shedding, and disease-related mortality affected outbreak dynamics in starkly contrasting ways. This effect appeared to be linked to the population-level distribution of each respective host trait, with factors such as skewness and zero-inflation influencing how variation in each trait affected outbreak dynamics. Here, we discuss the traits of individuals that posed the greatest transmission risk and why they pose less risk in diverse susceptible populations. We also analyse the potential effects of "supersponges",

that represent no transmission risk to the susceptible population, and reflect on the broader implications of these results for mitigating the spread of disease in other host-pathogen systems.

To limit the pathogen transmission from high-risk individuals often requires expensive and continuous monitoring by experts. Focussing on classes of high-risk individuals is a more pragmatic approach to reducing the effect of heterogeneity in transmission potential, requiring less intensive monitoring protocols (Drewe, 2009; VanderWaal and Ezenwa, 2016). Additionally, as classes of individuals are identified using ranges of physiological or behavioural traits, classes are potentially more generalisable to other host-pathogen systems (e.g. sex, social dominance). In theoretical experiment 1, males from the RAL-818 genetic background were not only more likely to start an outbreak of infectious disease, but these outbreaks were also more severe than in other populations. This suggests these males represent a class of individuals with a high transmission risk. Interestingly, high-risk males are seen in a number of host-pathogen systems (Ferrari et al., 2004; Grear et al., 2009). While high-risk male classes can be produced by a range of traits pertaining to sex-specific ecology or physiology, their occurrence across systems is likely driven by sexual selection shaping male traits affecting transmission (Zuk and McKean, 1996). For example, in the yellow-necked mouse, *Apodemus flavicollis*, males are thought to be a high-risk class due to a range of sex differences in their immune response, home range and contact rates (Ferrari et al., 2004). Moreover, as male *Drosophila* exhibit a number of other traits with the potential to alter their transmission potential, such as male-male fighting (Baxter et al., 2015), the transmission risk of RAL-818 males could increase further.

High-risk individuals, such as superspreaders, present a danger to current methods of disease control because they are capable of starting outbreaks of infectious disease that are difficult to predict and amplifying them once transmission begins (Craft and Caillaud, 2011; Keiser et al., 2017). This has driven pre-emptively identifying high-risk individuals to the forefront of epidemiology and disease

ecology. However, in the second theoretical experiment we conducted, we found that starting outbreaks with individuals that differed in transmission potential, did not affect outbreak dynamics in diverse susceptible populations. The diversity of the susceptible population acted as an effective buffer to disease transmission, through low-competency individuals surrounding high-risk individuals. This effectively isolates high-risk individuals from the rest of the population. Our results suggest outbreaks are not solely driven by the traits of rare, high-risk individuals, but are also affected by the traits of the susceptible population. This finding overtly reflects a major strength of host diversity, increased protection from pathogens and parasites (Lively, 2010; Ostfeld and Keesing, 2012). This finding bears a number of similarities with the broad observation that host diversity is central to the spread of disease (Altermatt and Ebert, 2008; Lively, 2010; Ostfeld and Keesing, 2012). The importance of host diversity to disease transmission is exemplified by the rapid spread of disease in crop monocultures due to crops being comprised of individuals with similar susceptibility (Mitchell et al., 2002; Pilet et al., 2006; Zhu et al., 2000). A diverse host population exposes disease-causing agents to different environments that can apply unique selection pressures which prevent their proliferation. Similar transmission dynamics have also been observed in laboratory populations of the social spider, *Stegodyphus dumicola*, where transmission of a bacterial pathogen was affected by the boldness of the index case and the individuals it interacted with (Keiser et al., 2017). Together with our results, these findings do not suggest diversity in the susceptible population is a universal buffer to the effects of between-individual heterogeneity in disease transmission. Instead, this work highlights the necessity to characterise population diversity in the context of social interactions and networks as these may determine the relevance of this diversity. There are many traits across systems that bias social interactions, for example, such as sexual receptivity or personality type (Keiser et al., 2016). Should these traits bias contact between transmission classes, this may explain why social and contact networks rarely match.

Extreme phenotypes often play a key role in between-individual heterogeneity in disease transmission. However, being a relative term, 'extreme' phenotypes are defined by population-level variation. Constraining population-level variation in the amount of virus shed following infection to the population mean increased outbreak likelihood and severity. This was likely a result of the huge zero-inflation of the distribution of virus shedding, where many infected individuals did not shed virus. These individuals, previously termed 'supersponges' (Barron et al., 2015), represent the left-most extreme of the population distribution, and bore no transmission risk. 'Supersponges' present a potential explanation to the widely observed 20-80 rule across a range of host-pathogen and parasite systems, where, during an outbreak of infectious disease, 20% of hosts are responsible for 80% of transmission (Woolhouse et al., 1997). While some of the individuals that do not transmit infection may simply not get any transmission opportunity, others may be supersponges and therefore incapable of transmitting disease.

An important caveat of our results is that because we did not measure social aggregation, virus shedding and lifespan simultaneously we cannot account for how they might covary in individuals. We therefore allow them to co-occur in hosts randomly, which may contradict associations produced in nature or predicted by hypotheses (Réale et al., 2010; Vazquez-Prokopec et al., 2016). This is particularly true for how we derived contact between susceptible and infected individuals from empirical data on social aggregation. An important caveat of contact in our simulations is that it is derived from social aggregation arenas containing 10-12 flies and measuring 55mm wide. We considered populations of 1000 individuals whose social network was derived by scaling-up the aggregation of considerably smaller populations. This approach was necessitated by the experimental demands of measuring social aggregation (Simon et al., 2012). However, it would be pertinent to experimentally test how *Drosophila* social aggregation changes with population size, particularly as this is a common issue where empirical data is integrated with *in silico* work. Moreover, larger populations often have different structures and more modular social networks which have been shown to facilitate or prevent the spread

of disease (Nunn et al., 2015; Sah et al., 2017). As our social aggregation data comes from Petri dishes containing only males or females from a single genetic background, we cannot account for how aggregation might change in more diverse and larger populations. Additionally, as very little is known about DCV transmission, the distance DCV transmission was able to occur over was inherently arbitrary, despite this issue being accounted for by threshold radius. Understanding how distance affects pathogen transmission or definitions of what constitutes a contact is a hugely influential relationship which is poorly described in many host-pathogen systems (White et al., 2018). The high levels of control in laboratory populations of *Drosophila* offer an ideal system to characterise this relationship.

Our work bears a number of consequences for understanding how between-individual heterogeneity in disease transmission is determined and how it could affect outbreak dynamics. We show that variation in key traits in individuals can dramatically affect population-level transmission, surmounting to genetic and sex-specific variation in transmission potential. Importantly, the influence of this variation is dramatically affected by susceptible population diversity and the distribution of population-level variation. These results support the observations of other systems that suggest the traits of susceptible individuals can exert significant influence over transmission. This is particularly relevant to populations with low genetic diversity, such as agricultural monocultures, as this lack of diversity increases the risk of explosive outbreaks (Pilet et al., 2006; Wallace and Wallace, 2015; Zhu et al., 2000). This result also applies to organisms with structured populations, such as familial herds, or nests, as this structure could produce localised groups of high-risk individuals. Our work posits the merits of integrating data collected in highly controlled laboratory experiments with simulations capable of extrapolating this information to larger populations. Simulation experiments may also help generate hypotheses of particular interest in a laboratory setting (Restif et al., 2012). Given that there are many other protocols available to the *D. melanogaster* model system, as well as many other systems, our holistic approach provides an ideal tool to

furthering our understanding of how rare, high-risk individuals affect population-level transmission dynamics.

Chapter 6: General Discussion

6.1 Overview

The primary aim of this thesis was to characterise the effect of broad sources of variation underlying between-individual heterogeneity in disease transmission, using *D. melanogaster* as a host and DCV as a pathogen. While *D. melanogaster* has been used to study key aspects of transmission such as virus shedding (Habayeb et al., 2009a), this thesis is the first body of work to consider multiple behavioural and physiological traits together and to integrate them into a simulation framework that relates them to disease transmission. With the exception of chapter 2, which tests the effect of infection on avoidance behaviours, this thesis focussed on the effect of genetic and sex-specific variation on transmission heterogeneity. My studies have considered the effect of genetic and sex-specific variation on multiple behaviours, physiological traits and outcomes of infection. Combining these patterns of variation has revealed genetic and sex-specific variation in transmission potential capable of influencing population-level disease transmission dynamics. Here, I summarise the findings of these experiments and discuss their implications and limitations, before finally commenting on directions for future research.

In Chapter 2, I tested the effect of infection on infection avoidance during larval foraging and adult oviposition. I found that larvae did not distinguish between infectious and non-infectious carcasses during cannibalistic foraging, and that this was not affected by infection. Conversely, during oviposition, females exhibited a preference for non-infectious sites that was affected by infection as well as the site's available resources. I suggest that infection avoidance during oviposition-site choice accounts for the absence of such avoidance during larval foraging as mothers determine the environment in which larvae forage and grow.

The experiments of Chapter 3 tested the effect of infection, as well as sex-specific, and genetic variation, on locomotor activity and social aggregation over two

separate experiments. I identified a male-specific decrease in social aggregation following DCV infection that is potentially explained by the costs incurred via male-male aggression being exacerbated by infection. I also observed broad patterns of genetic variation in social aggregation and locomotor activity, which taken together with the male-specific decrease in social aggregation suggest the potential for sex-specific and genetic variation in disease transmission.

Chapter 4 is also comprised of two experiments, the first, measured late-stage infection outcomes: lifespan following infection and viral load at death (VLAD), while the second measured virus shedding and viral load during the first 3 days of infection. Both experiments measured the effect of mating as well as, sex-specific and genetic variation on these outcomes of infection using males and females from the same genetic backgrounds as the social aggregation experiments in Chapter 3. These experiments show complex patterns of genetic and sex-specific variation in all infection outcomes, with the exception of VLAD which is not affected by sex or genetic background. I combine the data on lifespan and virus shedding, with the previously collected data on social aggregation to provide a simple estimate of individual variation in transmission, *V*. Analysis of *V*, revealed significant genetic and sex-specific variation in transmission potential that explained ~15% of between-individual heterogeneity.

In Chapter 5, I used an epidemiological model and measured several transmission parameters after constraining the diversity of theoretical populations or variation in virus shedding, lifespan and social aggregation was limited. Susceptible population diversity had a major impact on transmission dynamics, with low-risk individuals acting as effective buffer to transmission when connected to high-risk individuals. Constraining variation in virus shedding, lifespan and social aggregation revealed these components were affecting population-level transmission dynamics in starkly contrasting ways. The epidemiological model described in chapter 5 provides

evidence of sex-specific and genetic variation in individual disease transmission that is capable of influencing population-level transmission dynamics.

6.2 Key Findings & Implications

The work described in my thesis has broken down heterogeneity in disease transmission into behavioural and physiological sources of variation and consider how they are affected by genetic and sex-specific key sources of variation. My fifth chapter is a consolidation of these findings, using virus shedding, social aggregation and lifespan following infection to inform epidemiological models. In the section below, I discuss some of my thesis' main findings and their broader implications on our understanding of heterogeneity in disease transmission. I focus on the importance of genetic variation to disease transmission potential and the necessity of considering multiple host traits, with a particular emphasis on behaviour.

6.1.1 Genetic Variation Contributes to Transmission Heterogeneity

While genetic variation has been observed in a broad range of the traits that affect disease transmission, very few studies have identified a genetic component to variation in disease transmission. This thesis has demonstrated the potential importance of genetic variation in transmission heterogeneity through two frameworks that estimate disease transmission. Significant genetic variation in disease transmission suggests an individual's transmission potential is, at least partially, encoded by its genes. This has two major implications.

Firstly, identifying the genes involved in, or associated with, disease transmission would enable high-risk individuals to be identified before outbreaks of infectious disease occur. *D. melanogaster* is particularly amenable to GWAS, a tool particularly proficient at identifying major-effect genes (Mackay et al., 2012). However, given the involvement of highly variable infection outcomes, and multiple behavioural, and physiological traits in producing transmission heterogeneity, methods better able to

identify minor-effect genes (QTL mapping) and epistatic interactions (restricted partitioning methods; RPM) may be more appropriate (Culverhouse et al., 2004; Korte and Farlow, 2013; Mackay, 2001). Once identified, genes involved in disease transmission would provide tools to further study and characterise transmission heterogeneity using *D. melanogaster*. The epidemiological models in Chapter 5 suggest the traits of the index case are not always sufficient to produce an outbreak of disease. High or low transmission-risk mutants, however, could be used to test the extent to which transmission network position does limit initial outbreak dynamics and whether this can be overcome in certain criteria. Given the homology of certain genes between *D. melanogaster* and vertebrates (Ferreira et al., 2014; Lemaitre et al., 1996), transmission gene homologues may offer useful insights into transmission heterogeneity in other host-pathogen systems that cannot be studied in the lab.

The second major implication of detecting genetic variation in disease transmission is the potential for selection to produce transmission heterogeneity. Transmission is an integral part of pathogen and parasite fitness, which suggests there would be a selective advantage to infecting high-risk hosts. Selection pressures favouring the infection of high-risk hosts however, could interact with or be offset by host variation in infection exposure risk. Understanding the selective pressures that produce higher levels of transmission would also affect the predictability of transmission heterogeneity to change over time. Artificial selection could be used in variety of settings to lower individual's transmission risks. Selecting for lower disease transmission in crop agriculture, for example, could prove essential as crop monocultures are often vulnerable to rapid disease spread (Pilet et al., 2006; Zhu et al., 2000). It is important to note however that the viability of selecting for traits that reduce disease transmission depends on the traits being selected for not affecting crop production.

6.1.2 Sex-Specific Variation Contributes to Transmission Heterogeneity

Many studies of disease transmission report sex-specific variation, and apportion this to particular differences in male and female ecology, such as reduced immunity

(Adelman and Hawley, 2017; Mills et al., 2010) or more connected contact networks (Grear et al., 2009). Males were generally observed to have greater values of *V* and simulated all-male populations experienced outbreaks that infected more individuals than all-female populations. Male-bias in transmission suggests that the decrease in male social aggregation following infection is overshadowed by males shedding more virus. This sex difference suggests that the sexually dimorphic traits of *D. melanogaster* would serve as useful, non-intrusive identifiers of transmission risk.

Not only does the sex-difference in transmission provide a potentially viable marker of transmission risk but it may also point to differences in sexual selection underlying transmission heterogeneity. One possible explanation for the male-specific increase in social aggregation, for example, is the increased costs of male-male aggression being exacerbated by DCV infection. Similarly, differences in virus shedding between male and female *D. melanogaster*, may be a result of sexual dimorphism in gut morphology (Regan et al., 2016). The sexual dimorphism seen in a range of other traits (Apger-McGlaughon and Wolfner, 2013; Kubiak and Tinsley, 2017; Vale and Jardine, 2015) may help characterise many other factors that affect transmission heterogeneity. The sex-difference we see in transmission potential and a number of its underlying traits demonstrate a major limitation of only studying traits in female *D. melanogaster* (Bou Sleiman et al., 2015; Habayeb et al., 2009a; Longdon et al., 2011; Magwire et al., 2012). While sex-differences are not always relevant to the hypotheses of these studies, ignoring males overlooks an important source of variation. For example, the study that originally identified the involvement of *pastrel* in DCV susceptibility only used females (Magwire et al., 2012). My data on lifespan following infection however, suggests that males survive DCV infection longer than females.

6.1.3 It is Important to Measure Variation in Multiple Traits to Characterise Transmission Heterogeneity

In order to understand heterogeneity in disease transmission it is essential to consider a broad range of behavioural and physiological traits as well as a number of outcomes of infection. Overlooking the influence of particular traits on transmission could lead to inaccurate estimates of heterogeneity. This is evident in the various sex-differences seen across the behavioural and physiological traits I measured. The male-specific decrease in social aggregation, for example, would suggest males pose less transmission risk than females. Conversely, the reverse is suggested by the greater male lifespan following infection. This is also evident even within the behavioural traits I measured, some of which (e.g. infection avoidance during oviposition-site choice) are only relevant to females, and others only have effects on males (e.g. social aggregation). Interestingly, the relative importance of variation in certain traits to outbreak simulations where population-level variation was constrained to the mean appeared to be determined by the distribution of that trait through the population. The gaussian distribution of lifespan following infection meant that constraining variation to the mean had very little effect on transmission dynamics. Conversely, as a result of removing zero-inflation, constraining variation virus shedding resulted in dramatic increases in outbreak severity. This suggests that in addition to measuring multiple traits, it is also essential to characterise population-level variation in order to identify extreme phenotypes that may have disproportionate effects on transmission.

6.3 Limitations

The work in this thesis provides a number of useful insights to understanding transmission heterogeneity, however it is important to recognise some of this work's limitations. Here, I summarise the main limitations that should be addressed by future research.

6.3.1 Population Structure

Measuring social aggregation in groups comprised of the same sex and genetic background allows estimations of the effect of these sources of variation on social aggregation, however, this experimental design cannot account for population structure. This may affect how relatable the results of my experiments in Chapter 5 are to wild systems where population structure is commonplace. A number of innate or learned biases can promote individuals to socialise or aggregate with particular conspecifics and avoid others (Durisko et al., 2014; Perkins et al., 2008; Philippe et al., 2016). Biases in social interactions can promote or prevent transmission, depending on the traits of individuals that infected hosts interact with. Genetic variation has been shown to affect group size preference in adult *D. melanogaster* (Saltz, 2011) and in larvae, the genotypic composition of groups changes with group size (Philippe et al., 2016).

Mating-associated behaviours will arise in populations containing males and females and will introduce a number of potentially significant biases to social interactions between and within sexes. In *D. melanogaster*, males compete for access to females through fighting behaviour (Baxter et al., 2015). Alongside the short-term increase in male-male interactions this behaviour may produce longer lasting consequences for social interactions with losing these bouts having long-lasting consequences for behaviour such as reduced fighting and social isolation (Trannoy et al., 2015). Given their increased access to females, males that win bouts of aggression may represent key spreaders of disease. Courtship and copulation introduce further complexity and biases to interactions between males and females. Not only do sexual interactions increase contact between males and females, but copulation also incurs several female-biased costs (Short et al., 2012) which may promote transmission. Conversely, sexual interactions can promote female immunity, with courtship signals from males having been shown to upregulate the immune gene *Turandot M* in the presence of a sexually-transmitted fungal pathogen (Zhong et al., 2013).

6.3.2 Within-Individual Variation in Disease Transmission

In Chapter 3, I measured social aggregation 30 minutes after adding flies to a petri dish, this measure does not account for how social aggregation might change over time. Similarly, in Chapter 4, while I measured virus shedding and viral load at different time points, due to destructive sampling, these variables were only measured once per individual. By simultaneously measuring virus shedding and viral load I was able to test whether the amount of virus shed by an individual was affected by how much was growing inside it, but this design obscures how virus shedding and load changes within individuals over time. Within-individual variation in these traits may have a number of effects on how transmission potentials change over time and is thought to be another major source of transmission heterogeneity (Chen et al., 2013). Within-individual variation represents a particularly significant problem to disease control methods as it requires more regular and vigilant monitoring of infected individuals. Patterns of within-individual variation are particularly important to pathogen or parasite shedding and have been identified in a number of species (Brawner III and Hill, 1999; Chen et al., 2013; Gopinath et al., 2014; Martinaud et al., 2009; Misof, 2004; Schiffer et al., 2014; Tavalire et al., 2016; Williams et al., 2014). Unfortunately, as viral load sampling is inherently destructive, there is very little scope to measure within individual variation. However, how virus shedding changes over time could be measured by transferring individuals into new food-containing Eppendorf tubes every 24 hours.

Estimations of V did not account for this change in virus shedding over time and while simulated outbreaks of DCV occurred over multiple time steps, virus shedding was assumed to be constant within individuals. Within-individual variation in virus shedding may therefore have a number of important consequences for estimations of V and simulations of outbreaks in theoretical populations.

The male-specific decrease in social aggregation following infection also demonstrates the potential for within-individual variation in social aggregation. As outbreak simulations utilised a static contact network, where connections did not

change over time, within-individual variation in social aggregation could affect transmission heterogeneity. The static contact network design may also have led to more individuals becoming isolated from transmission. When they are infected, isolated individuals are unable to transmit infection to the susceptible populations and when they are susceptible, they are protected from transmission. Isolation from transmission can occur when connected nodes die from infection or shed no DCV. Dynamic contact networks, where connections between individuals are more transient and freely form would prevent isolation from the transmission networks and likely begin to capture the importance of within-individual variation in social aggregation. How social aggregation changes over time could be measured in a similar experiment to that seen in Chapter 3, but where aggregation is measured at multiple time points. Video tracking software is also becoming increasingly used to understand complex social interactions in both *D. melanogaster* (Dankert et al., 2009; Slawson et al., 2009), and a number of other species (Hong et al., 2015; Mersch et al., 2013; Weissbrod et al., 2013).

6.3.3 Covariation between Behavioural and Physiological Traits

Covariation and coupled heterogeneities are thought to be central to producing high-risk individuals (Hawley and Altizer, 2011; Vazquez-Prokopec et al., 2016; White et al., 2018). Individuals that exhibit high pathogen or parasite shedding while also being gregarious, for example, are likely to expose many susceptible individuals to infection. Covariation between relatively disparate traits may also be expected in certain cases, with selection suggested to favour individuals that heavily invest in either short or long-term strategies (Réale et al., 2010).

To measure virus shedding, social aggregation and lifespan following infection, required three separate experimental designs, preventing the measurement of these three traits in the same individual. As a result, my data offers no information on the extent of phenotypic covariation. In epidemiological models, I assumed no covariation between virus shedding, social aggregation and lifespan following infection and drew values randomly from treatment group data. Mathematical

models have demonstrated the potential for positive and negative covariation between behavioural and physiological traits to affect outbreak dynamics (White et al., 2018). The nature of covariation between virus shedding, social aggregation and lifespan following infection could be addressed similarly, by incorporating phenotypic covariation into how individual traits are assigned. However, simulating these patterns of covariation entails making further assumptions regarding the extent of covariation and does not account for potential genetic or sex-specific differences. This could be overcome by designing experiments that measure multiple traits in the same individuals and using the levels of covariation observed to inform how individual traits are produced in simulations.

6.3.4 Variation in the Risk of Acquiring Infection

This thesis has focussed on understanding variation in traits that, when present in infected individuals, increases their ability to infect susceptible individuals. However, a number of traits can increase a susceptible individual's likelihood of becoming infected (Dizney and Dearing, 2013; Drewe, 2009). The traits that increase infection risk are often distinct from those that promote transmission. As transmission is determined by infected and susceptible individuals, competent 'receivers' present another source of heterogeneity to transmission that acts as a major barrier to our understanding. Despite many studies reporting measures of disease prevalence in wild host-pathogen systems (Doeschl-Wilson et al., 2011; Ferrari et al., 2004; Rhines, 2013; Williams et al., 2014), like transmission heterogeneity, relatively little is known regarding infection exposure heterogeneity.

Infection exposure is primarily determined by an individual's ability to avoid and subsequently resist infection. Given that a high infection risk is inherently more detrimental to individual fitness than a high transmission risk, competent receivers should only be favoured by selection under certain circumstances. For example, bolder individuals are more prone to exploring novel or new environments, in laboratory populations of wood frog (*Lithobates sylvaticus*) larvae, bolder individuals experienced higher levels of parasitism (Koprivnikar Janet et al., 2012). In the social

spider, *Stegodyphus dumicola*, boldness has also been shown to promote transmission between nestmates (Keiser et al., 2016). Future work should aim to identify and characterise the traits that influence an individual's risk of acquiring infection from an infected individual alongside the traits that promote transmission.

6.4 Future Directions

A major contribution of this thesis is the establishment of *D. melanogaster* as a model to study disease transmission and its heterogeneity. Alongside this work, emerging techniques to study behaviour in the fruit fly (Itskov et al., 2014; Ja et al., 2007; Slawson et al., 2009; Wayland et al., 2014) offer an avenue of research with immense untapped potential. In this section, I discuss future directions of research that, using *D. melanogaster*, would further characterise individual heterogeneity in disease transmission and address limitations of my experiments.

6.4.1 Directly Measure Disease Transmission

Directly measuring the effect of genetic and sex-specific variation on disease transmission would provide useful information on the efficacy of my estimations of *V* and outbreak simulations. In order to be relatable to the measures taken in this thesis' experiments, this should be done using DCV. A barrier to understanding how DCV transmission dynamics change over time is that successful transmission is detected through destructive sampling, which can only be taken at a single time point. Host-pathogen systems with visual symptoms of infection would address this issue by using visual symptoms to infer transmission (Almberg et al., 2015; Anacleto et al., 2019). However, while DCV can result in abdominal swelling in the later stages of infection, there are no clear visual symptoms of early stage infection (Chtarbanova et al., 2014). I attempted to develop a non-invasive method of detecting DCV infection that used a mutant that fluoresced following activation of the viral immune gene, *vir-1* (Dostert et al., 2005). This protocol eventually failed however as GFP activation was relatively unreliable following faecal-oral transmission. Enhancing the expression of GFPs following *vir-1* activation may address this problem. Another

potential solution that would enable disease transmission to be measured in *D. melanogaster* is the use of fluorescent pathogens, which has been employed to validate transmission of a cuticular bacterial pathogen within social spider (*Stegodyphus dumicola*) colonies (Keiser et al., 2016).

6.4.2 Test the Effect of Disease Control Methods

Many disease control methods prevent transmission by altering infectiousness, contact rate or the duration of the infectious period. Using an epidemiological model, I tested the effect of limiting variation in virus shedding, social aggregation and lifespan following infection to the mean of the population. Manipulating variation in ways that approximate disease control methods could offer an effective way of identifying the control strategies that best respond to transmission heterogeneity. Quarantine control methods, for example, could be simulated by limiting the network connections of infected individuals after a certain number of time steps have elapsed. A key finding of simulations was the relatively negligible effect of constraining lifespan following infection to the population mean. I attributed this to the normality of the population-level distribution of lifespan following infection as removing individuals lying to either side of the mean affects both extremes equally, whereas more dispersed distributions affect one extreme greater than the other. However, given that in many cases, modern medicine acts to extend the life of sick patients or promote host tolerance (Vale et al., 2014), the effect of lifespan following infection on transmission may be affected by incorporating treatment methods into simulations. The effect of increasing tolerance could be modelled by incorporating 'treated' individuals with an increased lifespan following infection. It would also be possible to then test how the potentially negative effects of increased tolerance could be accounted for by other disease control methods such as quarantining, which would work to reduce infected individual contact rates.

6.4.3 Characterise the Route of Transmission and its Determinants

A more mechanistic understanding of how traits affect the process of disease transmission is crucial to understanding the importance of the genetic and sex-

specific variation I have reported in this thesis. It is currently not known, for example, how closely flies need to aggregate to facilitate transmission and how long DCV is able to survive in the environment outside of a host. My simulations of DCV outbreaks accounted for uncertainty in pathogen viability and transmission distance by considering multiple levels of variation in both of these factors. However, it is important to note that there may be important sources of variation in these factors that may influence pathogen transmission. For example, DCV shed by hosts of a particular genetic background or sex may be more viable than those shed by other hosts. Variation in infectious potential has been seen in the bacterial pathogen *Holospora undulata* where relaxed selection pressures on transmission increased the pathogen's ability to infect its host (Magalon et al., 2010). Alongside detailing these relatively external factors, future research should aim to characterise the importance of particular factors relative to one another. For example, in my estimations of disease transmission I assumed that variation in virus shedding, social aggregation and lifespan following infection were equally important. However, were virus shedding found to be more central to transmission heterogeneity than social aggregation, extreme shedding phenotypes will be far more influential to transmission than extreme aggregation phenotypes. Understanding the factors that drive disease transmission relative to one another will provide essential information regarding which traits control methods should prioritise.

Reference List

Adamo, S.A., Bartlett, A., Le, J., Spencer, N., Sullivan, K., 2010. Illness-induced anorexia may reduce trade-offs between digestion and immune function. *Animal Behaviour*. 79, pp.3–10.

Adelman, J.S., Hawley, D.M., 2017. Tolerance of infection: A role for animal behavior, potential immune mechanisms, and consequences for parasite transmission. *Hormones and Behavior.* 86, pp.79-86

Afonina, I., Ankoudinova, I., Mills, A., Lokhov, S., Huynh, P., Mahoney, W., 2007. Primers with 5' flaps improve real-time PCR. *BioTechniques.* 43 pp.770, 772, 774.

Ahmad, M., Chaudhary, S.U., Afzal, A.J., Tariq, M., 2015. Starvation-Induced Dietary Behaviour in *Drosophila melanogaster* Larvae and Adults. S*cientific reports*. 5, 14285.

Albeny-Simoes, D., Murrell, E.G., Elliot, S.L., Andrade, M.R., Lima, E., Juliano, S.A., Vilela, E.F., 2014. Attracted to the enemy: *Aedes aegypti* prefers oviposition sites with predator-killed conspecifics. *Oecologia.* 175, pp.481–492.

Alma, C.R., Gillespie, D.R., Roitberg, B.D., Goettel, M.S., 2010. Threat of Infection and Threat-Avoidance Behavior in the Predator *Dicyphus hesperus* Feeding on Whitefly Nymphs Infected with an Entomopathogen. *Journal of Insect Behavior*. 23, pp.90–99.

Almberg, E.S., Cross, P.C., Dobson, A.P., Smith, D.W., Metz, M.C., Stahler, D.R., Hudson, P.J., 2015. Social living mitigates the costs of a chronic illness in a cooperative carnivore. *Ecology Letters*. 18, pp.660–667.

Alpers, M.P., 2008. The epidemiology of kuru: monitoring the epidemic from its peak to its end. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 363, pp.3707–3713.

Altermatt, F., Ebert, D., 2008. Genetic diversity of *Daphnia magna* populations enhances resistance to parasites. *Ecology Letters*. 11, pp.918–928.

Altizer, S., Dobson, A., Hosseini, P., Hudson, P., Pascual, M., Rohani, P., 2006. Seasonality and the dynamics of infectious diseases. *Ecology Letters*. 9, pp. 467–484.

Altizer, S., Nunn, C.L., Thrall, P.H., Gittleman, J.L., Antonovics, J., Cunningham, A.A., Dobson, A.P.A., Ezenwa, V., Jones, K.E., Pedersen, A.B., Poss, M., Pulliam, J.R.C., 2003. Social organization and parasite risk in mammals: Integrating theory and empirical studies. *Annual Review of Ecology Evolution and Systematics*. 34, pp.517–547.

Anacker, A.M.J., Beery, A.K., 2013. Life in groups: the roles of oxytocin in mammalian sociality. *Front Behav Neurosci.* 7, pp.1-10

Anacleto, O., Cabaleiro, S., Villanueva, B., Saura, M., Houston, R.D., Woolliams, J.A., Doeschl-Wilson, A.B., 2019. Genetic differences in host infectivity affect disease spread and survival in epidemics. *Scientific Reports.* 9, 4924.

Anderson, B.B., Scott, A., Dukas, R., 2016. Social behavior and activity are decoupled in larval and adult fruit flies. *Behavioral Ecology*. 27, pp.820–828.

Anderson, R.M., May, R.M., 1992. Infectious Diseases of Humans: Dynamics and Control. Oxford University Press, Oxford, New York.

Anderson, R.M., May, R.M., 1981. The Population Dynamics of Microparasites and Their Invertebrate Hosts. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 291, pp.451–524.

Andersson, M., Simmons, L.W., 2006. Sexual selection and mate choice. *Trends in Ecology & Evolution*. 21, pp.296–302.

Apger-McGlaughon, J., Wolfner, M.F., 2013. Post-mating change in excretion by mated *Drosophila melanogaster* females is a long-term response that depends on sex peptide and sperm. *Journal of Insect Physiology*. 59, pp.1024–1030.

Apidianakis, Y., Rahme, L.G., 2009. *Drosophila melanogaster* as a model host for studying *Pseudomonas aeruginosa* infection. *Nature Protocols*. 4, pp.1285–1294.
Arnold, P.A., Johnson, K.N., White, C.R., 2013. Physiological and metabolic consequences of viral infection in Drosophila melanogaster. *The Journal of Experimental Biology*. 216, pp.3350–3357.
Arnold, P.A., White, C.R., Johnson, K.N., 2015. *Drosophila melanogaster* does not exhibit a behavioural fever response when infected with *Drosophila* C virus. *Journal of General Virology*. 96, pp.3667–3671.
Ashby, B., Boots, M., 2015. Coevolution of parasite virulence and host mating strategies. *Proceedings of the National Academy of Sciences of the United States of America*. 112, pp.13290–13295.
Ayroles, J.F., Carbone, M.A., Stone, E.A., Jordan, K.W., Lyman, R.F., Magwire, M.M., Rollmann, S.M., Duncan, L.H., Lawrence, F., Anholt, R.R.H., Mackay, T.F.C., 2009. Systems genetics of complex traits in *Drosophila melanogaster*. *Nature Genetics*. 41, pp. 299–307.
Babin, A., Kolly, S., Schneider, F., Dolivo, V., Zini, M., Kawecki, T.J., 2014. Fruit flies learn to avoid odours associated with virulent infection. *Biology letters.* 10, 20140048.
Baier, A., Wittek, B., Brembs, B., 2002. *Drosophila* as a new model organism for the neurobiology of aggression? *Journal of Experimental Biology*. 205, pp.1233–1240.
Bandi, C., Dunn, A.M., Hurst, G.D.D., Rigaud, T., 2001. Inherited microorganisms, sex-specific virulence and reproductive parasitism. *Trends in Parasitology*. 17, pp.88–94.
Barron, D.G., Gervasi, S.S., Pruitt, J.N., Martin, L.B., 2015. Behavioral competence: How host behaviors can interact to influence parasite transmission risk. *Current Opinion in Behavioral Sciences*. 6, pp.35–40.
Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*. 67.
Battesti, M., Moreno, C., Joly, D., Mery, F., 2012. Spread of Social Information and Dynamics of Social Transmission within *Drosophila* Groups. *Current Biology*. 22, pp.309–313.
Baxter, C.M., Barnett, R., Dukas, R., 2015. Aggression, mate guarding and fitness in male fruit flies. *Animal Behaviour*. 109, pp.235–241.
Behringer, D.C., Butler, M.J., Shields, J.D., 2006. Ecology: avoidance of disease by social lobsters. *Nature.* 441, pp.421–421.
Beldomenico, P.M., Begon, M., 2010. Disease spread, susceptibility and infection intensity: vicious circles? *Trends Ecol. Evol. (Amst.)*. 25, pp.21–27.
Berdoy, Webster J. P., Macdonald D. W., 2000. Fatal attraction in rats infected with *Toxoplasma gondii*. *Proceedings of the Royal Society of London. Series B: Biological Sciences*. 267, pp.1591–1594.
Bier, E., 2005. *Drosophila*, the golden bug, emerges as a tool for human genetics. *Nature Reviews Genetics*. 6, pp.9-23
Bier, E., Guichard, A., 2012. Deconstructing host-pathogen interactions in *Drosophila*. *Dis Model Mech*. 5, pp.48–61.
Blaustein, L., 1999. Oviposition Site Selection in Response to Risk of Predation: Evidence from Aquatic Habitats and Consequences for Population Dynamics and Community Structure, in: Wasser, S.P. (Ed.), Evolutionary Theory and Processes: Modern Perspectives. Springer Netherlands, pp. pp.441–456.
Bolzoni, L., Real, L., Leo, G.D., 2007. Transmission Heterogeneity and Control Strategies for Infectious Disease Emergence. *PLOS ONE*. 2, e747.
Boomsma, J.J., Baer, B., Heinze, J., 2004. The evolution of male traits in social insects. *Annu. Rev. Entomol.* 50, pp.395–420.
Boots, M., 1998. Cannibalism and the stage-dependent transmission of a viral pathogen of the Indian meal moth, *Plodia interpunctella*. E*cological Entomology*. 23, pp.118–122.
Bou Sleiman, M.S., Osman, D., Massouras, A., Hoffmann, A.A., Lemaitre, B., Deplancke, B., 2015. Genetic, molecular and physiological basis of

variation in *Drosophila* gut immunocompetence. *Nature Communications*. 6, pp.7829–7829.
Bouwman, K.M., Hawley, D.M., 2010. Sickness behaviour acting as an evolutionary trap? Male house finches preferentially feed near diseased conspecifics. *Biology letters*. 6, pp.462–5.
Bradley, C.A., Altizer, S., 2005. Parasites hinder monarch butterfly flight: implications for disease spread in migratory hosts. *Ecology Letters*. 8, pp.290–300.
Brawner III, W.R., Hill, G.E., 1999. Temporal variation in shedding of coccidial oocysts: implications for sexual-selection studies. *Can. J. Zool.* 77, pp.347–350.
Breiman, L., 2001. Random forests. *Machine Learning.* 45, pp.5–32.
Bretman, A., Westmancoat, J.D., Gage, M.J.G., Chapman, T., 2013. Costs and Benefits of Lifetime Exposure to Mating Rivals in Male *Drosophila melanogaster*. *Evolution*. 67, pp.2413–2422.
Brooks-Pollock, E., Roberts, G.O., Keeling, M.J., 2014. A dynamic model of bovine tuberculosis spread and control in Great Britain. *Nature.* 511, pp.228–231.
Brown, C.R., Brown, M.B., 2000. Heritable basis for choice of group size in a colonial bird. *Proceedings of the National Academy of Sciences of the United States of America.* 97, pp.14825–14830.
Brownstein, J.S., Hett, E., O'Neill, S.L., 2003. The potential of virulent Wolbachia to modulate disease transmission by insects. *Journal of Invertebrate Pathology*. 84, pp.24–29.
Bruns, E., Carson, M., May, G., 2012. Pathogen and host genotype differently affect pathogen fitness through their effects on different life-history stages. *BMC Evol Biol.* 12, 135.
Buchon, N., Silverman, N., Cherry, S., 2014. Immunity in *Drosophila melanogaster* — from microbial recognition to whole-organism physiology. *Nature Reviews Immunology* 14, pp.796–810.
Budischak, S.A., Sakamoto, K., Megow, L.C., Cummings, K.R., Urban, J.F., Ezenwa, V.O., 2015. Resource limitation alters the consequences of co-infection for both hosts and parasites. *International Journal for Parasitology*. 45, pp.455–463.
Camiletti, A.L., Thompson, G.J., 2016. *Drosophila* as a Genetically Tractable Model for Social Insect Behavior. *Front. Ecol. Evol.* 4 pp.1-9
Capitanio, J.P., Abel, K., Mendoza, S.P., Blozis, S.A., McChesney, M.B., Cole, S.W., Mason, W.A., 2008. Personality and serotonin transporter genotype interact with social context to affect immunity and viral set-point in simian immunodeficiency virus disease. *Brain Behav. Immun*. 22, pp.676–689.
Cartar, R.V., 1992. Morphological Senescence and Longevity: An Experiment Relating Wing Wear and Life Span in Foraging Wild Bumble Bees. *Journal of Animal Ecology*. 61, pp.225–231.
Centers for Disease Control and Prevention (CDC), 2003. Severe acute respiratory syndrome--Singapore, 2003. *MMWR Morb. Mortal. Wkly. Rep.* 52, pp.405–411.
Chambers, M.C., Jacobson, E., Khalil, S., Lazzaro, B.P., 2014. Thorax Injury Lowers Resistance to Infection in *Drosophila melanogaster*. *Infect Immun*. 82, pp.4380–4389.
Charlat Sylvain, Davies Neil, Roderick George K, Hurst Gregory D.D, 2007. Disrupting the timing of Wolbachia-induced male-killing. *Biology Letters*. 3, pp.154–156.
Chen, S., Lee, A.Y., Bowens, N.M., Huber, R., Kravitz, E.A., 2002. Fighting fruit flies: A model system for the study of aggression. *Proceedings of the National Academy of Sciences of the United States of America*. 99, pp.5664–5668.
Chen, S., Sanderson, M., Lanzas, C., 2013. Investigating effects of between- and within-host variability on *Escherichia coli* O157 shedding pattern and transmission. *Preventive Veterinary Medicine*. 109, pp.47–57.
Chiyo, P.I., Grieneisen, L.E., Wittemyer, G., Moss, C.J., Lee, P.C., Douglas-Hamilton, I., Archie, E.A., 2014. The Influence of Social Structure, Habitat,

and Host Traits on the Transmission of *Escherichia coli* in Wild Elephants. *PLOS ONE*. 9, e93408.
Chtarbanova, S., Lamiable, O., Lee, K.-Z., Galiana, D., Troxler, L., Meignin, C., Hetru, C., Hoffmann, J.A., Daeffler, L. 7, Imler, J.-L., 2014. *Drosophila* C virus systemic infection leads to intestinal obstruction. *Journal of Virology*. 88, pp.14057–14069.
Clark, P.J., Evans, F.C., 1954. Distance to Nearest Neighbor as a Measure of Spatial Relationships in Populations. *Ecology*. 35, pp.445–453.
Colinet H., Renault D., 2012. Metabolic effects of CO_2 anaesthesia in *Drosophila melanogaster*. *Biology Letters*. 8, pp.1050–1054.
Córdoba-Aguilar, A., González-Tokman, D., González-Santoyo, I. (Eds.), 2018. Insect Behavior: From Mechanisms to Ecological and Evolutionary Consequences. *Oxford University Press*, Oxford, New York.
Cornet, S., Bichet, C., Larcombe, S., Faivre, B., Sorci, G., 2014. Impact of host nutritional status on infection dynamics and parasite virulence in a bird-malaria system. *J Anim Ecol*. 83, pp.256–265.
Craft, M.E., 2015. Infectious disease transmission and contact networks in wildlife and livestock. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*. 370, p.20140107.
Craft, M.E., Caillaud, D., 2011. Network Models: An Underutilized Tool in Wildlife Epidemiology? *Interdisciplinary Perspectives on Infectious Diseases.* 11, pp. 1-12
Cremer, S., Armitage, S.A.O., Schmid-Hempel, P., 2007. Social Immunity. *Current Biology*. 17, pp.R693–R702.
Cross, P.C., Johnson, P.L.F., Lloyd-Smith, J.O., Getz, W.M., 2007. Utility of R0 as a predictor of disease invasion in structured populations. *J R Soc Interface*. 4, pp.315–324.
Culverhouse, R., Klein, T., Shannon, W., 2004. Detecting epistatic interactions contributing to quantitative traits. *Genet. Epidemiol*. 27, pp.141–152.
Curtis, V., de Barra, M., Aunger, R., 2011. Disgust as an adaptive system for disease avoidance behaviour. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* 366, pp.389–401.
Curtis, V.A., 2014. Infection-avoidance behaviour in humans and other animals. *Trends in Immunology*. 35, pp.457–464.
Cutler, D.R., Edwards, T.C., Beard, K.H., Cutler, A., Hess, K.T., Gibson, J., Lawler, J.J., 2007. Random forests for classification in ecology. *Ecology.* 88, pp.2783–2792.
Dankert, H., Wang, L., Hoopfer, E.D., Anderson, D.J., Perona, P., 2009. Automated Monitoring and Analysis of Social Behavior in *Drosophila*. *Nat Methods*. 6, pp.297–303.
de Bekker, C., Ohm, R.A., Loreto, R.G., Sebastian, A., Albert, I., Merrow, M., Brachmann, A., Hughes, D.P., 2015. Gene expression during zombie ant biting behavior reflects the complexity underlying fungal parasitic behavioral manipulation. *BMC Genomics*. 16, pp.1-23.
de Bono, M., Bargmann, C.I., 1998. Natural variation in a neuropeptide Y receptor homolog modifies social behavior and food response in C. elegans. *Cell.* 94, pp.679–89.
de Roode, J.C., Lefèvre, T., 2012. Behavioral Immunity in Insects. *Insects.* 3, pp.789–820.
Dhandapani, N., Jayaraj, S., Rabindra, R.J., 1993. Cannibalism on nuclear polyhedrosis virus infected larvae by *Heliothis armigera* (Hubn.) and its effect on viral infection. *International Journal of Tropical Insect Science*. 14, pp.427–430.
Dierick, H. a, Greenspan, R.J., 2006. Molecular analysis of flies selected for aggressive behavior. *Nature genetics*. 38, pp.1023–31.
Dierick, H.A., 2007. A method for quantifying aggression in male *Drosophila melanogaster*. *Nature protocols*. 2, pp.2712–8.
Dizney, L., Dearing, M.D., 2013. The role of behavioural heterogeneity on infection patterns: implications for pathogen transmission. *Anim Behav*. 86, pp.1-15.
Doeschl-Wilson, A.B., Davidson, R., Conington, J., Roughsedge, T., Hutchings, M.R., Villanueva, B., 2011. Implications of host genetic variation on the risk

and prevalence of infectious diseases transmitted through the environment. *Genetics.* 188, pp.683–693.
Dostert, C., Jouanguy, E., Irving, P., Troxler, L., Galiana-Arnoux, D., Hetru, C., Hoffmann, J.A., Imler, J.-L., 2005. The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of *Drosophila*. *Nature immunology*. 6, pp.946–53.
Drewe, J.A., 2009. Who infects whom? Social networks and tuberculosis transmission in wild meerkats. *Proc. Biol. Sci.* 277, pp.633–642.
Drewe, J.A., Eames, K.T.D., Madden, J.R., Pearce, G.P., 2011. Integrating contact network structure into tuberculosis epidemiology in meerkats in South Africa: Implications for control. *Prev. Vet. Med.* 101, pp.113–120.
Duneau, D., Ebert, D., 2012. Host Sexual Dimorphism and Parasite Adaptation. *PLOS Biology*. 10, e1001271.
Duneau, D., Ferdy, J.B., Revah, J., Kondolf, H., Ortiz, G.A., Lazzaro, B.P., Buchon, N., 2017. Stochastic variation in the initial phase of bacterial infection predicts the probability of survival in *D. melanogaster*. eLife 6, e28298–e28298.
Durisko, Z., Anderson, B., Dukas, R., 2014. Adult fruit fly attraction to larvae biases experience and mediates social learning. *The Journal of experimental biology*. 217, pp.1193–1197.
Durisko, Z., Dukas, R., 2013. Attraction to and learning from social cues in fruitfly larvae. *Proceedings of the Royal Society B: Biological Sciences*. 280, pp.20131398–20131398.
Ebstein, R.P., Israel, S., Chew, S.H., Zhong, S., Knafo, A., 2010. Genetics of Human Social Behavior. *Neuron*. 65, pp.831–844.
Edwards, A.C., Rollmann, S.M., Morgan, T.J., Mackay, T.F.C., 2006. Quantitative Genomics of Aggressive Behavior in *Drosophila melanogaster*. *PLoS Genetics*. 2, pp.1386–1395.
Edwards, J.L., Apicella, M.A., 2004. The Molecular Mechanisms Used by Neisseria gonorrhoeae To Initiate Infection Differ between Men and Women. *Clinical Microbiology Reviews*. 17, pp.965–981.
Elderd, B.D. 2013. Developing Models of Disease Transmission: Insights from Ecological Studies of Insects and Their Baculoviruses. *PLoS Pathogens*. 9, e1003372–e1003372.
Elvira, S., Williams, T., Caballero, P., 2010. Juvenile Hormone Analog Technology: Effects on Larval Cannibalism and the Production of *Spodoptera exigua* (Lepidoptera: Noctuidae) Nucleopolyhedrovirus. *Journal of Economic Entomology*. 103, pp.577–582.
Escobedo-Bonilla, C.M., Alday-Sanz, V., Wille, M., Sorgeloos, P., Pensaert, M.B., Nauwynck, H.J., 2008. A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus. *Journal of Fish Diseases*. 31, pp.1–18.
Ezenwa, V.O., Archie, E.A., Craft, M.E., Hawley, D.M., Martin, L.B., Moore, J., White, L., 2016a. Host behaviour–parasite feedback: an essential link between animal behaviour and disease ecology. *Proc. R. Soc. B*. 283, 20153078.
Farrington, C.P., Whitaker, H.J., Unkel, S., Pebody, R., 2013. Correlated Infections: Quantifying Individual Heterogeneity in the Spread of Infectious Diseases. *Am J Epidemiol*. 177, pp.474–486.
Feldman, R., Monakhov, M., Pratt, M., Ebstein, R.P., 2016. Oxytocin Pathway Genes: Evolutionary Ancient System Impacting on Human Affiliation, Sociality, and Psychopathology. Biological Psychiatry, Oxytocin and Psychiatry: From DNA to Social Behavior. *Biological Psychiatry*. 79, pp.174–184.
Fellous, S., Duncan, A.B., Quillery, E., Vale, P.F., Kaltz, O., 2012. Genetic influence on disease spread following arrival of infected carriers. *Ecology Letters*. 15, pp.186–192.
Ferrari, N., Cattadori, I.M., Nespereira, J., Rizzoli, A., Hudson, P.J., 2004. The role of host sex in parasite dynamics: field experiments on the yellow-necked mouse *Apodemus flavicollis*. *Ecology Letters*. 7, pp.88–94.

Ferreira, Á.G., Naylor, H., Esteves, S.S., Pais, I.S., Martins, N.E., Teixeira, L., 2014. The Toll-Dorsal Pathway Is Required for Resistance to Viral Oral Infection in *Drosophila*. *PLoS Pathogens*. 10, e1004507–e1004507.
Fleming, G., 1872. Rabies and Hydrophobia: Their History, Nature, Causes, Symptoms, and Prevention. *Chapman and Hall.*
Folstad, I., Karter, A.J., 1992. Parasites, Bright Males, and the Immunocompetence Handicap. *The American Naturalist*. 139, pp.603–622.
Fontaine, A., Lequime, S., Moltini-Conclois, I., Jiolle, D., Leparc-Goffart, I., Jr, R.C.R., Lambrechts, L., 2018. Epidemiological significance of dengue virus genetic variation in mosquito infection dynamics. *PLOS Pathogens*. 14, e1007187.
Foo, Y.Z., Nakagawa, S., Rhodes, G., Simmons, L.W., 2017. The effects of sex hormones on immune function: a meta-analysis. *Biological Reviews*. 92, pp.551–571.
Forbes, L.B., 2000. The occurrence and ecology of Trichinella in marine mammals. *Veterinary Parasitology*. 93, pp.321–334.
Fouks, B., Lattorff, H.M.G. 2011. Recognition and Avoidance of Contaminated Flowers by Foraging Bumblebees (*Bombus terrestris*). *PLoS ONE*. 6, e26328–e26328.
Fredericksen, M.A., Zhang, Y., Hazen, M.L., Loreto, R.G., Mangold, C.A., Chen, D.Z., Hughes, D.P., 2017. Three-dimensional visualization and a deep-learning model reveal complex fungal parasite networks in behaviorally manipulated ants. *Proceedings of the National Academy of Sciences of the United States of America*.114, pp.12590–12595.
Gandon, S., Day, T., Metcalf, C.J.E., Grenfell, B.T., 2016. Forecasting Epidemiological and Evolutionary Dynamics of Infectious Diseases. *Trends in Ecology & Evolution*. 31, pp.776–788.
Garbutt, J.S., O'Donoghue, A.J.P., McTaggart, S.J., Wilson, P.J., Little, T.J., 2014. The development of pathogen resistance in *Daphnia magna*: implications for disease spread in age-structured populations. *J. Exp. Biol*. 217, pp.3929–3934.
Gervasi, S.S., Civitello, D.J., Kilvitis, H.J., Martin, L.B., 2015. The context of host competence: A role for plasticity in host-parasite dynamics. *Trends in Parasitology*. 31, pp.419–425.
Gill, D.M., 1977. Mechanism of action of cholera toxin. *Adv Cyclic Nucleotide Res*. 8, pp.85–118.
Gjedrem, T., Salte, R., Gjøen, H.M., 1991. Genetic variation in susceptibility of Atlantic salmon to furunculosis. *Aquaculture*. 97, pp.1–6.
Godfrey, S.S., 2013. Networks and the ecology of parasite transmission: A framework for wildlife parasitology. *International Journal for Parasitology: Parasites and Wildlife*. 2, pp.235–245.
Gomariz-Zilber, E., Thomas-Orillard, M., 1993. *Drosophila* C virus and *Drosophila* hosts: a good association in various environments. *Journal of Evolutionary Biology*. 6, pp.677–689.
Gopinath, S., Lichtman, J.S., Bouley, D.M., Elias, J.E., Monack, D.M., 2014. Role of disease-associated tolerance in infectious superspreaders. *Proceedings of the National Academy of Sciences of the United States of America*. 111, pp.15780–15785.
Govind, S., 2008. Innate immunity in *Drosophila*: Pathogens and pathways. *Insect Sci*. 15, pp.29–43.
Graham, A.L., Allen, J.E., Read, A.F., 2005. Evolutionary Causes and Consequences of Immunopathology. *Annual Review of Ecology, Evolution, and Systematics*. 36, pp.373–397.
Grassly, N.C., Fraser, C., 2008. Mathematical models of infectious disease transmission. *Nature reviews. Microbiology*. 6, pp.234–244.
Grassly, N.C., Fraser, C., 2006. Seasonal infectious disease epidemiology. *Proceedings of the Royal Society B: Biological Sciences*. 273, pp.2541–2550.
Grear, D.A., Luong, L.T., Hudson, P.J., 2012. Sex-biased transmission of a complex life-cycle parasite: why males matter. *Oikos.* 121, pp.1446–1453.

Grear, D.A., Perkins, S.E., Hudson, P.J., 2009. Does elevated testosterone result in increased exposure and transmission of parasites? *Ecology Letters*. 12, pp.528–537.
Grenfell, B.T., Anderson, R.M., 1985. The estimation of age-related rates of infection from case notifications and serological data. *J Hyg (Lond)*. 95, pp.419–436.
Grzybek, M., Bajer, A., Behnke-Borowczyk, J., Al-Sarraf, M., Behnke, J.M., 2015. Female host sex-biased parasitism with the rodent stomach nematode *Mastophorus muris* in wild bank voles (*Myodes glareolus*). *Parasitol Res*. 114, pp.523–533.
Gupta, V., Stewart, C., Rund, S.S.C., Monteith, K., Vale, P.F., 2017a. Costs and benefits of sub-lethal *Drosophila* C virus infection. *Journal of Evolutionary*
Gupta, V., Vale, P.F., 2017. Nonlinear disease tolerance curves reveal distinct components of host responses to viral infection. *R Soc Open Sci.* 4, pp.1-12
Gupta, V., Vasanthakrishnan, R.B., Siva-Jothy, J., Monteith, K.M., Brown, S.P., Vale, P.F., 2017. The route of infection determines Wolbachia antibacterial protection in *Drosophila*. *Proc Biol Sci*. 284, pp.1-9
Habayeb, M.S., Cantera, R., Casanova, G., Ekström, J.-O., Albright, S., Hultmark, D., 2009a. The *Drosophila* Nora virus is an enteric virus, transmitted via feces. *Journal of Invertebrate Pathology*. 101, pp.29–33.
Habayeb, M.S., Ekström, J.O., Hultmark, D., 2009b. Nora virus persistent infections are not affected by the RNAi machinery. *PLoS ONE*. 4, pp.4–8.
Hales, K.G., Korey, C.A., Larracuente, A.M., Roberts, D.M., 2015. Genetics on the Fly: A Primer on the *Drosophila* Model System. *Genetics.* 201, pp.815–842.
Hall, S.R., Sivars-Becker, L., Becker, C., Duffy, M.A., Tessier, A.J., Cáceres, C.E., 2007. Eating yourself sick: transmission of disease as a function of foraging ecology. *Ecology Letters*. 10, pp.207–218.
Hammock, E.A.D., Young, L.J., 2005. Microsatellite Instability Generates Diversity in Brain and Sociobehavioral Traits. *Science.* 308, pp.1630–1634.
Harabis, F., Dolný, A., Helebrandov, J., Rusková, T., 2015. Do egg parasitoids increase the tendency of *Lestes sponsa* (Odonata: Lestidae) to oviposit underwater? *European Journal of Entomology*. 112, pp.63–68.
Hart, B.L., 1988. Biological basis of the behavior of sick animals. *Neuroscience & Biobehavioral Reviews*. 12, pp.123–137.
Hawley, D.M., Altizer, S.M., 2011. Disease ecology meets ecological immunology: understanding the links between organismal immunity and infection dynamics in natural populations. *Functional Ecology*. 25, pp.48–60.
Hawley, D.M., Etienne, R.S., Ezenwa, V.O., Jolles, A.E., 2011. Does animal behavior underlie covariation between hosts' exposure to infectious agents and susceptibility to infection? Implications for disease dynamics. *Integrative and comparative biology*. 51, pp.528–39.
Heil, M., 2016. Host Manipulation by Parasites: Cases, Patterns, and Remaining Doubts. *Front. Ecol. Evol.* 4.
Hollis, B., Kawecki, T.J., Keller, L., 2015. No evidence that within-group male relatedness reduces harm to females in *Drosophila*. *Ecology and Evolution*. 5, pp.979–983.
Hong, W., Kennedy, A., Burgos-Artizzu, X.P., Zelikowsky, M., Navonne, S.G., Perona, P., Anderson, D.J., 2015. Automated measurement of mouse social behaviors using depth sensing, video tracking, and machine learning. *Proceedings of the National Academy of Sciences of the United States of America*.112, E5351–E5360.
Hooper, R.R., Reynolds, G.H., Jones, O.G., Zaidi, A., Wiesner, P.J., Latimer, K.P., Lester, A., Campbell, A.F., Harrison, W.O., Karney, W.W., Holmes, K.K., 1978. COHORT STUDY OF VENEREAL DISEASE. I: THE RISK OF GONORRHEA TRANSMISSION FROM INFECTED WOMEN TO MEN. *Am J Epidemiol*. 108, pp.136–144.
Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous Inference in General Parametric Models. *Biometrical Journal*. 50, pp.346–363.
Hoye, B., Fouchier, R., Klaassen, M., 2012. Host behaviour and physiology underpin individual variation in avian influenza virus infection in migratory

Bewick's swans. *Proceedings of the Royal Society B: Biological Sciences*. 279, pp.529–534.
Hughes, D.P., Brodeur, J., Thomas, F., 2012. Host Manipulation by Parasites. Oxford University Press.
Huszar, T., Imler, J., 2008. *Drosophila* Viruses and the Study of Antiviral Host-Defense, in: Advances in Virus Research. *Academic Press*. pp. 227–265.
Israel, S., Lerer, E., Shalev, I., Uzefovsky, F., Reibold, M., Bachner-Melman, R., Granot, R., Bornstein, G., Knafo, A., Yirmiya, N., Ebstein, R.P., 2008. Molecular genetic studies of the *arginine vasopressin 1a receptor* (*AVPR1a*) and the oxytocin receptor (OXTR) in human behaviour: from autism to altruism with some notes in between. *Prog. Brain Res.* 170, pp.435–449.
Itskov, P.M., Moreira, J.-M., Vinnik, E., Lopes, G., Safarik, S., Dickinson, M.H., Ribeiro, C., 2014. Automated monitoring and quantitative analysis of feeding behaviour in *Drosophila*. *Nature Communications*. 5, 4560.
Izhar, R., Routtu, J., Ben-Ami, F., 2015. Host age modulates within-host parasite competition. *Biol. Lett.* 11, 20150131.
Ja, W.W., Carvalho, G.B., Mak, E.M., de la Rosa, N.N., Fang, A.Y., Liong, J.C., Brummel, T., Benzer, S., 2007. Prandiology of *Drosophila* and the CAFE assay. *Proceedings of the National Academy of Sciences of the United States of America*. 104, pp.8253–8256.
Jennings, B.H., 2011. *Drosophila* – a versatile model in biology & medicine. *Materials Today*. 14, pp.190–195.
Johnson, R.W., 2002. The concept of sickness behavior: a brief chronological account of four key discoveries. *Veterinary Immunology and Immunopathology, Fossum S. I.* 87, pp.443–450.
Jousset, F.-X., Bergoin, M., Revet, B., 1977. Characterization of the *Drosophila* C Virus. *J Gen Virol.* 34, pp.269–283.
Kacsoh, B.Z., Lynch, Z.R., Mortimer, N.T., Schlenke, T.A., 2013. Fruit flies medicate offspring after seeing parasites. *Science*. 339, pp.947–50.
Kaltz, O., Shykoff, J.A., 2001. Male and female *Silene latifolia* plants differ in per-contact risk of infection by a sexually transmitted disease. *Journal of Ecology*. 89, pp.99–109.
Kapun, M., Nolte, V., Flatt, T., Schlötterer, C., 2010. Host Range and Specificity of the *Drosophila* C Virus. *PLoS One*. 5.
Kavaliers, M., Choleris, E., Ågmo, A., Pfaff, D.W., 2004. Olfactory-mediated parasite recognition and avoidance: linking genes to behavior. *Hormones and Behavior, Olfaction, Sex, and Behavior*. 46, pp.272–283.
Kazlauskas, N., Klappenbach, M., Depino, A.M., Locatelli, F.F., 2016. Sickness Behavior in Honey Bees. *Front Physiol*. 7.
Keebaugh, E.S., Schlenke, T.A., 2014. Insights from natural host–parasite interactions: The Drosophila model. *Developmental & Comparative Immunology, Drosophila Immunity*. 42, pp.111–123.
Keeling, M.J., Rohani, P., 2007. Chapter One Contact Tracing Operates by Refining the Targeting of Other Control Measures. 1.4. What Are Mathematical Models?
Keiser, Carl N., Howell, K.A., Pinter-Wollman, N., Pruitt, J.N., 2016. Personality composition alters the transmission of cuticular bacteria in social groups. *Biology Letters*. 12, 20160297.
Keiser, Carl N., Pinter-Wollman, N., Augustine, D.A., Ziemba, M.J., Hao, L., Lawrence, J.G., Pruitt, J.N., 2016b. Individual differences in boldness influence patterns of social interactions and the transmission of cuticular bacteria among group-mates. *Proceedings of the Royal Society B: Biological Sciences*. 283, pp.20160457–20160457.
Keiser, C.N., Pinter-Wollman, N., Ziemba, M.J., Kothamasu, K.S., Pruitt, J.N., 2017. The index case is not enough: Variation among individuals, groups and social networks modify bacterial transmission dynamics. *Journal of Animal Ecology*. pp.1–10.
Kelly, C.D., Stoehr, A.M., Nunn, C., Smyth, K.N., Prokop, Z.M., 2018. Sexual dimorphism in immunity across animals: a meta-analysis. *Ecol. Lett.* 21, pp.1885–1894.

Kiesecker, J.M., Skelly, D.K., Beard, K.H., Preisser, E., 1999. Behavioral reduction of infection risk. *Proceedings of the National Academy of Sciences of the United States of America*. 96, pp.9165–9168.
Kiffner, C., Lödige, C., Alings, M., Vor, T., Rühe, F., 2011. Body-mass or sex-biased tick parasitism in roe deer (*Capreolus capreolus*)? A GAMLSS approach. *Med. Vet. Entomol.* 25, pp.39–45.
Klämbt, C., Jacobs, J.R., Goodman, C.S., 1991. The midline of the drosophila central nervous system: A model for the genetic analysis of cell fate, cell migration, and growth cone guidance. *Cell.* 64, pp.801–815.
Klarsfeld, A., Leloup, J.-C., Rouyer, F., 2003. Circadian rhythms of locomotor activity in Drosophila. *Behavioural Processes, Drosophila behaviour: concepts and tools.* 64, pp.161–175.
Klemme, I., Karvonen, A., 2016. Learned parasite avoidance is driven by host personality and resistance to infection in a fish–trematode interaction. *Proceedings of the Royal Society of London B: Biological Sciences*. 283.
Kocher, S.D., Mallarino, R., Rubin, B.E.R., Yu, D.W., Hoekstra, H.E., Pierce, N.E., 2018. The genetic basis of a social polymorphism in halictid bees. *Nature Communications.* 9, p.4338.
Koets, A.P., Adugna, G., Janss, L.L.G., van Weering, H.J., Kalis, C.H.J., Wentink, G.H., Rutten, V.P.M.G., Schukken, Y.H., 2000. Genetic Variation of Susceptibility to Mycobacterium avium subsp. paratuberculosis Infection in Dairy Cattle. *Journal of Dairy Science*. 83, pp.2702–2708.
Kolluru, G.R., Grether, G.F., Dunlop, E., South, S.H., 2009. Food availability and parasite infection influence mating tactics in guppies (*Poecilia reticulata*). *Behav Ecol.* 20, pp.131–137.
Konopka, R.J., Benzer, S., 1971. Clock mutants of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*. 68, pp.2112–6.
Koprivnikar Janet, Gibson Chris H., Redfern Julia C., 2012. Infectious personalities: behavioural syndromes and disease risk in larval amphibians. *Proceedings of the Royal Society B: Biological Sciences*. 279, pp.1544–1550.
Korte, A., Farlow, A., 2013. The advantages and limitations of trait analysis with GWAS: a review. *Plant methods*. 9, p.29.
Kounatidis, I., Ligoxygakis, P., 2012. *Drosophila* as a model system to unravel the layers of innate immunity to infection. *Open Biology*. 2, 120075.
Kubiak, M., Tinsley, M.C., 2017. Sex-Specific Routes to Immune Senescence in *Drosophila melanogaster*. *Scientific Reports*. 7, 10417.
Kuo, T.-H., Williams, J.A., 2014. Increased sleep promotes survival during a bacterial infection in *Drosophila*. *Sleep* 37, pp.1077–86.
Kurz, C.L., Charroux, B., Chaduli, D., Viallat-Lieutaud, A., Royet, J., 2017. Peptidoglycan sensing by octopaminergic neurons modulates *Drosophila oviposition*. *eLife* 6.
Kutzer, M. a. M., Kurtz, J., Armitage, S. a. O., 2018. Genotype and diet affect resistance, survival, and fecundity but not fecundity tolerance. *J. Evol. Biol.* 31, pp.159–171.
Lambrechts, L., 2011. Quantitative genetics of *Aedes aegypti* vector competence for dengue viruses: towards a new paradigm? *Trends in Parasitology*. 27, pp.111–114.
Lass Sandra, Hudson Peter J., Thakar Juilee, Saric Jasmina, Harvill Eric, Albert Réka, Perkins Sarah E., 2013. Generating super-shedders: co-infection increases bacterial load and egg production of a gastrointestinal helminth. *Journal of The Royal Society Interface*. 10, 20120588.
Lazzaro, B.P., Little, T.J., 2009. Immunity in a variable world. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences.* 364, pp.15–26.
Lazzaro, B.P., Sackton, T.B., Clark, A.G., 2006. Genetic variation in Drosophila melanogaster resistance to infection: a comparison across bacteria. *Genetics.* 174, pp.1539–54.
Lea, A.J., Blumstein, D.T., Wey, T.W., Martin, J.G.A., 2010. Heritable victimization and the benefits of agonistic relationships. *Proceedings of the National*

Academy of Sciences of the United States of America. 107, pp.21587–21592.
Lee, Y.C.G., Yang, Q., Chi, W., Turkson, S.A., Du, W.A., Kemkemer, C., Zeng, Z.-B., Long, M., Zhuang, X., 2017. Genetic Architecture of Natural Variation Underlying Adult Foraging Behavior That Is Essential for Survival of *Drosophila melanogaster*. *Genome Biology and Evolution*. 9, pp.1357–1369.
Lefèvre, T., Chiang, A., Kelavkar, M., Li, H., Li, J., de Castillejo, C.L.F., Oliver, L., Potini, Y., Hunter, M.D., de Roode, J.C., 2012. Behavioural resistance against a protozoan parasite in the monarch butterfly. *J Anim Ecol*. 81, pp.70–79.
Lefèvre, T., de Roode, J.C., Kacsoh, B.Z., Schlenke, T.A., 2011a. Defence strategies against a parasitoid wasp in *Drosophila*: fight or flight? *Biology Letters*.
Lefèvre, T., Williams, A.J., de Roode, J.C., 2011b. Genetic variation in resistance, but not tolerance, to a protozoan parasite in the monarch butterfly. *Proc. Biol. Sci.* 278, pp.751–759.
Lemaitre, B., Hoffmann, J., 2007. The Host Defense of *Drosophila melanogaster*. *Annual Review of Immunology*. 25, pp.697–743.
Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., Hoffmann, J.A., 1996. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell.* 86, pp.973–983.
Lewis, E., 2014. A new standard food medium. 1960 *Drosophila* Information Service. *Cold Spring Harb Protoc*. 2014, pdb.rec081414.
Li, J., Blakeley, D., Smith, R.J., 2011. The Failure of R_0. *Comput Math Methods Med* 2011.
Li, Y., Yu, I.T.S., Xu, P., Lee, J.H.W., Wong, T.W., Ooi, P.L., Sleigh, A.C., 2004. Predicting super spreading events during the 2003 severe acute respiratory syndrome epidemics in Hong Kong and Singapore. *American journal of epidemiology*. 160, pp.719–28.
Life Technologies, 2011. MagMAX-96 Total RNA Isolation Kit User Guide. *Carlsbad, CA USA: Life Technologies Corporation*.
Lihoreau, M., Clarke, I.M., Buhl, J., Sumpter, D.J.T., Simpson, S.J., 2016a. Collective selection of food patches in Drosophila. *Journal of Experimental Biology*. 219, pp.668–675.
Lihoreau, M., Poissonnier, L.-A., Isabel, G., Dussutour, A., 2016b. *Drosophila* females trade off good nutrition with high-quality oviposition sites when choosing foods. *Journal of Experimental Biology*. 219.
Lim, R.S., Eyjlfsdttir, E., Shin, E., Perona, P., Anderson, D.J., 2014. How food controls aggression in *Drosophila*. *PLoS ONE* 9.
Lin, C.-C., Prokop-Prigge, K.A., Preti, G., Potter, C.J., 2015. Food odors trigger *Drosophila* males to deposit a pheromone that guides aggregation and female oviposition decisions. *Elife* 4.
Litvinova, E.A., Goncharova, E.P., Zaydman, A.M., Zenkova, M.A., Moshkin, M.P., 2010. Female Scent Signals Enhance the Resistance of Male Mice to Influenza. *PLOS ONE* 5, e9473.
Liu, W., Liang, X., Gong, J., Yang, Z., Zhang, Y.-H., Zhang, J.-X., Rao, Y., 2011. Social regulation of aggression by pheromonal activation of *Or65a* olfactory neurons in *Drosophila*. *Nature Neuroscience*. 14, pp.896–902.
Lively, C.M., 2010. The Effect of Host Genetic Diversity on Disease Spread. *The American Naturalist*. 175, E149–E152.
Llewellyn, D., Brown, G.P., Thompson, M.B., Shine, R., 2011. Behavioral Responses to Immune-System Activation in an Anuran (the Cane Toad, *Bufo marinus*): Field and Laboratory Studies. *Physiological and Biochemical Zoology*. 84, pp.77–86.
Lloyd-Smith James O., Getz Wayne M., Westerhoff Hans V., 2004. Frequency–dependent incidence in models of sexually transmitted diseases: portrayal of pair–based transmission and effects of illness on contact behaviour. *Proceedings of the Royal Society of London. Series B: Biological Sciences*. 271, pp.625–634.

Lloyd-Smith, J.O., George, D., Pepin, K.M., Pitzer, V.E., Pulliam, J.R.C., Dobson, A.P., Hudson, P.J., Grenfell, B.T., 2009. Epidemic dynamics at the human-animal interface. *Science*. 326, pp.1362–1367.
Lloyd-Smith, James O, Schreiber, S., Kopp, P., Getz, W., 2005. Superspreading and the effect of individual variation on disease emergence. *Nature.* 438, pp.355–359.
Lloyd-Smith, J.O., Schreiber, S.J., Getz, W.M., 2006. Moving beyond averages: individual-level variation in disease transmission, in: Gumel, A.B., Castillo-Chavez, C., Mickens, R.E., Clemence, D.P. (Eds.), Contemporary Mathematics. *American Mathematical Society, Providence, Rhode Island.* pp. 235–258.
Lloyd-Smith, J O, Schreiber, S.J., Kopp, P.E., Getz, W.M. 2005. Superspreading and the effect of individual variation on disease emergence. *Nature.* 438, pp.355–9.
Long, T.A.F., Pischedda, A., Stewart, A.D., Rice, W.R., 2009. A Cost of Sexual Attractiveness to High-Fitness Females. *PLOS Biology*. 7, e1000254.
Longdon, B., Hadfield, J.D., Webster, C.L., Obbard, D.J., Jiggins, F.M., 2011. Host Phylogeny Determines Viral Persistence and Replication in Novel Hosts. *PLoS Pathogens*. 7, e1002260–e1002260.
Loosli, C.G., Lemon, H.M., Robertson, O.H., Appel, E., 1943. Experimental Air-Borne Influenza Infection. I. Influence of Humidity on Survival of Virus in Air. *Proceedings of the Society for Experimental Biology and Medicine*. 53, pp.205–206.
Lopes, P.C., Block, P., König, B., 2016. Infection-induced behavioural changes reduce connectivity and the potential for disease spread in wild mice contact networks. *Scientific Reports*. 6, 31790.
Louis, M., de Polavieja, G., 2017. Collective Behavior: Social Digging in *Drosophila* Larvae. *Current Biology* 27, R1010–R1012.
Luong, L.T., Grear, D.A., Hudson, P.J., 2009. Male hosts are responsible for the transmission of a trophically transmitted parasite, Pterygodermatites peromysci, to the intermediate host in the absence of sex-biased infection. *International Journal for Parasitology*. 39, pp.1263–1268.
Mackay, T.F.C., 2001. Quantitative trait loci in *Drosophila*. *Nature Reviews Genetics*. 2, 11.
Mackay, T.F.C., Richards, S., Stone, E.A., Barbadilla, A., Ayroles, J.F., Zhu, D., Casillas, S., Han, Y., Magwire, M.M., Cridland, J.M., Richardson, M.F., Anholt, R.R.H., Barrón, M., Bess, C., Blankenburg, K.P., Carbone, M.A., Castellano, D., Chaboub, L., Duncan, L., Harris, Z., Javaid, M., Jayaseelan, J.C., Jhangiani, S.N., Jordan, K.W., Lara, F., Lawrence, F., Lee, S.L., Librado, P., Linheiro, R.S., Lyman, R.F., Mackey, A.J., Munidasa, M., Muzny, D.M., Nazareth, L., Newsham, I., Perales, L., Pu, L.-L., Qu, C., Ràmia, M., Reid, J.G., Rollmann, S.M., Rozas, J., Saada, N., Turlapati, L., Worley, K.C., Wu, Y.-Q., Yamamoto, A., Zhu, Y., Bergman, C.M., Thornton, K.R., Mittelman, D., Gibbs, R.A., 2012. The *Drosophila melanogaster* Genetic Reference Panel. *Nature.* 482, pp.173–8.
Magalon, H., Nidelet, T., Martin, G., Kaltz, O., 2010. Host growth conditions influence experimental evolution of life history and virulence of a parasite with vertical and horizontal transmission. *Evolution.* 64, pp.2126–2138.
Magwire, M.M., Fabian, D.K., Schweyen, H., Cao, C., Longdon, B., Bayer, F., Jiggins, F.M., 2012. Genome-Wide Association Studies Reveal a Simple Genetic Basis of Resistance to Naturally Coevolving Viruses in *Drosophila melanogaster*. *PLoS Genetics* 8.
Marineli, F., Tsoucalas, G., Karamanou, M., Androutsos, G., 2013. Mary Mallon (1869-1938) and the history of typhoid fever. *Ann Gastroenterol*. 26, pp.132–134.
Martinaud, G., Billaudelle, M., Moreau, J., 2009. Circadian variation in shedding of the oocysts of *Isospora turdi* (Apicomplexa) in blackbirds (*Turdus merula*): An adaptative trait against desiccation and ultraviolet radiation. *International Journal for Parasitology.* 39, pp.735–739.
Matthews, L., Low, J.C., Gally, D.L., Pearce, M.C., Mellor, D.J., Heesterbeek, J. a P., Chase-Topping, M., Naylor, S.W., Shaw, D.J., Reid, S.W.J., Gunn, G.J.,

Woolhouse, M.E.J., 2006. Heterogeneous shedding of Escherichia coli O157 in cattle and its implications for control. *Proceedings of the National Academy of Sciences of the United States of America*. 103, pp.547–552.
May, R.M., Anderson, R.M., 1987. Transmission dynamics of HIV infection. *Nature.* 326, pp.137–42.
McCallum, H., Fenton, A., Hudson, P.J., Lee, B., Levick, B., Norman, R., Perkins, S.E., Viney, M., Wilson, A.J., Lello, J., Mccallum, H., 2017. Breaking beta: deconstructing the parasite transmission function. *Philosophical Transactions B*. 372, 20160084.
McMullan, R., Anderson, A., Nurrish, S., 2012. Behavioral and Immune Responses to Infection Require Gαq- RhoA Signaling in *C. elegans*. *PLOS Pathogens*. 8, e1002530.
Medzhitov, R., Preston-Hurlburt, P., Janeway, C.A., 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388, 394.
Meisel, J.D., Kim, D.H., 2014. Behavioral avoidance of pathogenic bacteria by *Caenorhabditis elegans*. *Trends in immunology*. 35, pp.465–70.
Meisel, J.D., Panda, O., Mahanti, P., Schroeder, F.C., Kim, D.H., 2014. Chemosensation of Bacterial Secondary Metabolites Modulates Neuroendocrine Signaling and Behavior of *C. elegans*. *Cell.* 159, pp.267–280.
Merkling, S.H., van Rij, R.P., 2015. Analysis of resistance and tolerance to virus infection in *Drosophila*. *Nature protocols*. 10, pp.1084–97.
Mersch, D.P., Crespi, A., Keller, L., Robinson, G.E., Robinson, E.J.H., Feinerman, O., Franks, N.R., Jandt, J.M., Dornhaus, A., Smith, C.R., Toth, A.L., Suarez, A.V., Robinson, G.E., Johnson, B.R., Seeley, T.D., Vonshak, M., Shlagman, A., Rosvall, M., Bergstrom, C.T., Seid, M.A., Traniello, J.F.A., Lenoir, A., Mourier, J., Vercelloni, J., Planes, S., Lusseau, D., Blonder, B., Dornhaus, A., Shannon, P., Briscoe, A.D., Chittka, L., Traniello, J.F.A., Fortunato, S., 2013. Tracking individuals shows spatial fidelity is a key regulator of ant social organization. *Science*. 340, pp.1090–3.
Mideo, N., Alizon, S., Day, T., 2008. Linking within- and between-host dynamics in the evolutionary epidemiology of infectious diseases. *Trends Ecol. Evol. (Amst.)* 23, pp.511–517.
Mills, S.C., Grapputo, A., Jokinen, I., Koskela, E., Mappes, T., Poikonen, T., 2010. Fitness Trade-Offs Mediated by Immunosuppression Costs in a Small Mammal. *Evolution*. 64, pp.166–179.
Misof, K., 2004. Diurnal cycle of *Isospora* spp. oocyst shedding in Eurasian blackbirds (*Turdus merula*). *Can. J. Zool.* 82, pp.764–768.
Mitchell, C.E., Tilman, D., Groth, J.V., 2002. Effects of Grassland Plant Species Diversity, Abundance, and Composition on Foliar Fungal Disease. *Ecology.* 83, pp.1713–1726.
Moore, J., 2013. An overview of parasite-induced behavioral alterations - and some lessons from bats. *The Journal of experimental biology.* 216, pp.11–17.
Morgan, T.H., 1910. SEX LIMITED INHERITANCE IN *DROSOPHILA*. *Science* 32, 120–122.
Moroń, D., Witek, M., Woyciechowski, M., 2008. Division of labour among workers with different life expectancy in the ant *Myrmica scabrinodis*. *Animal Behaviour.* 75, 345–350.
Mussabekova, A., Daeffler, L., Imler, J.-L., 2017. Innate and intrinsic antiviral immunity in *Drosophila*. *Cellular and Molecular Life Sciences.* pp.1–16.
Neyen, C., Bretscher, A.J., Binggeli, O., Lemaitre, B., 2014. Methods to study *Drosophila immunity*. *Methods*. 68, pp.116–128.
Nilsen, S.P., Chan, Y.-B., Huber, R., Kravitz, E.A., 2004. Gender-selective patterns of aggressive behavior in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*.101, pp.12342–12347.
Nunn, C.L., Jordán, F., McCabe, C.M., Verdolin, J.L., Fewell, J.H., 2015. Infectious disease and group size: more than just a numbers game. *Philos Trans R Soc Lond B Biol Sci.* 370.

Ophir, A.G., Gessel, A., Zheng, D.-J., Phelps, S.M., 2012. Oxytocin receptor density is associated with male mating tactics and social monogamy. *Hormones and Behavior, Oxytocin, Vasopressin and Social Behavior.* 61, pp.445–453.
Orr, MatthewR., 1992. Parasitic flies (Diptera: Phoridae) influence foraging rhythms and caste division of labor in the leaf-cutter ant, *Atta cephalotes* (Hymenoptera: Formicidae). *Behavioral Ecology and Sociobiology*. 30, pp.395–402.
Ostfeld, R.S., Keesing, F., 2012. Effects of Host Diversity on Infectious Disease. *Annual Review of Ecology, Evolution, and Systematics*. 43, pp.157–182.
Pai, A., Yan, G., 2003. Effects of tapeworm infection on male reproductive success and mating vigor in the red flour beetle, *Tribolium castaneum*. *J. Parasitol.* 89, pp.516–521.
Palmer, W.H., Medd, N.C., Beard, P.M., Obbard, D.J., 2018. Isolation of a natural DNA virus of *Drosophila melanogaster*, and characterisation of host resistance and immune responses. *PLOS Pathogens*. 14, e1007050.
Pandey, U.B., Nichols, C.D., 2011. Human Disease Models in *Drosophila melanogaster* and the Role of the Fly in Therapeutic Drug Discovery. *Pharmacol Rev*. 63, pp.411–436.
Panteleev, D.Yu., Goryacheva, I.I., Andrianov, B.V., Reznik, N.L., Lazebny, O.E., Kulikov, A.M., 2007. The endosymbiotic bacterium Wolbachia enhances the nonspecific resistance to insect pathogens and alters behavior of *Drosophila melanogaster*. *Russ J Genet.* 43, pp.1066–1069.
Parker, B.J., Barribeau, S.M., Laughton, A.M., de Roode, J.C., Gerardo, N.M., 2011. Non-immunological defense in an evolutionary framework. *Trends in Ecology & Evolution*. 26, pp.242–248.
Parker, B.J., Elderd, B.D., Dwyer, G., 2010. Host behaviour and exposure risk in an insect-pathogen interaction. *Journal of Animal Ecology.* 79, pp.863–870.
Parker, K.J., Phillips, K.M., Kinney, L.F., Lee, T.M., 2001. Day length and sociosexual cohabitation alter central oxytocin receptor binding in female meadow voles (*Microtus pennsylvanicus*). *Behav. Neurosci.* 115, pp.1349–1356.
Patterson, J.E.H., Ruckstuhl, K.E., 2013. Parasite infection and host group size: a meta-analytical review. *Parasitology*. 140, pp.803–813.
Paull, S.H., Song, S., McClure, K.M., Sackett, L.C., Kilpatrick, A.M., Johnson, P.T., 2011. From superspreaders to disease hotspots: linking transmission across hosts and space. *Frontiers in Ecology and the Environment*. 10, pp.75–82.
Paull, S.H., Song, S., McClure, K.M., Sackett, L.C., Kilpatrick, A.M., Johnson, P.T.J., 2012. From superspreaders to disease hotspots: linking transmission across hosts and space. *Frontiers in ecology and the environment*. 10, pp.75–82.
Pearman, P.B., Garner, T.W.J., Straub, M., Greber, U.F., 2004. Response of the Italian agile frog (*Rana latastei*) to a Ranavirus, frog virus 3: a model for viral emergence in naïve populations. *J. Wildl. Dis*. 40, pp.660–669.
Penn, J.K.M., Zito, M.F., Kravitz, E.A., 2010. A single social defeat reduces aggression in a highly aggressive strain of *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*. 107, pp.12682–6.
Pereira, H.S., Sokolowski, M.B., 1993. Mutations in the larval foraging gene affect adult locomotory behavior after feeding in *Drosophila melanogaster. Proceedings of the National Academy of Sciences of the United States of America*. 90, pp.5044–5046.
Perkins, S.E., Ferrari, M.F., Hudson, P.J., 2008. The effects of social structure and sex-biased transmission on macroparasite infection. *Parasitology.* 135, pp.1561–1569.
Pfeiffenberger, C., Lear, B.C., Keegan, K.P., Allada, R., 2010. Processing Sleep Data Created with the *Drosophila* Activity Monitoring (DAM) System. *Cold Spring Harb Protoc*, pdb.prot5520.
Pfennig, D.W., Loeb, M.L.G., Collins, J.P., 1991. Pathogens as a factor limiting the spread of cannibalism in tiger salamanders. *Oecologia.* 88, pp.161–166.

Philippe, A.-S., Jeanson, R., Pasquaretta, C., Rebaudo, F., Sueur, C., Mery, F., 2016. Genetic variation in aggregation behaviour and interacting phenotypes in *Drosophila*. *Proceedings. Biological sciences*. 283, pp.20152967–20152967.
Pilet, F., Chacón, G., Forbes, G.A., Andrivon, D., 2006. Protection of Susceptible Potato Cultivars Against Late Blight in Mixtures Increases with Decreasing Disease Pressure. *Phytopathology*. 96, pp.777–783.
Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., Beutler, B., 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science.* 282, pp.2085–2088.
Ponton, F., Wilson, K., Holmes, A.J., Cotter, S.C., Raubenheimer, D., Simpson, S.J., 2013. Integrating nutrition and immunology: A new frontier. Journal of Insect Physiology, Immune Interactions Between Insects and Their Natural Antagonists: a Workshop Honoring Professor Stuart E. Reynolds. 59, pp.130–137.
Poulin, R., 1995. "Adaptive" changes in the behaviour of parasitized animals: a critical review. *Int. J. Parasitol*. 25, pp.1371–1383.
Quiring, R., Walldorf, U., Kloter, U., Gehring, W.J., 1994. Homology of the eyeless gene of *Drosophila* to the Small eye gene in mice and Aniridia in humans. *Science*. 265, pp.785–789.
Qureshi, T., Labes, R.E., Lambeth, M., Montgomery, H., Griffin, J.F.T., Mackintosh, C.G., 2000. Transmission of *Mycobacterium bovis* from experimentally infected ferrets to non-infected ferrets (*Mustela furo*). *New Zealand Veterinary Journal.* 48, pp.99–104.
Réale, D., Garant, D., Humphries, M.M., Bergeron, P., Careau, V., Montiglio, P.-O., 2010. Personality and the emergence of the pace-of-life syndrome concept at the population level. *Philos Trans R Soc Lond B Biol Sci.* 365, pp.4051–4063.
Regan, J., Khericha, M., Dobson, A.J., Bolukbasi, E., Rattanavirotkul, N., Partridge, L., 2016. Sex difference in pathology of the ageing gut mediates the greater response of female lifespan to dietary restriction. eLIFE 5, e10956.
Restif, O., Hayman, D.T.S., Pulliam, J.R.C., Plowright, R.K., George, D.B., Luis, A.D., Cunningham, A.A., Bowen, R.A., Fooks, A.R., O'Shea, T.J., Wood, J.L.N., Webb, C.T., 2012. Model-guided fieldwork: practical guidelines for multidisciplinary research on wildlife ecological and epidemiological dynamics. *Ecology Letters*. 15, pp.1083–1094.
Rhines, A.S., 2013. The role of sex differences in the prevalence and transmission of tuberculosis. Tuberculosis, Featuring Reports From The Tuberculosis Community Annotation Project Jamboree held at Virginia Tech, USA, March 7-8, 2012 93, pp.104–107.
Ridenhour, B., Kowalik, J.M., Shay, D.K., 2014. Unraveling R_0: Considerations for Public Health Applications. *Am J Public Health*. 104, e32–e41.
Rotiroti, D., Focà, A., Mastroeni, P., Fumarola, D., Nisticò, G., 1981. Behavioural and body temperature effects of meningococcal lipopolysaccharide after intraventricular injection in adult fowls *Gallus domesticus*. *Res Commun Chem Pathol Pharmacol*. 33, pp.395–402.
Rupprecht, C.E., Hanlon, C.A., Hemachudha, T., 2002. Rabies re-examined. *The Lancet Infectious Diseases*. 2, pp.327–343.
Rushmore, J., Caillaud, D., Matamba, L., Stumpf, R.M., Borgatti, S.P., Altizer, S., 2013. Social network analysis of wild chimpanzees provides insights for predicting infectious disease risk. *Journal of Animal Ecology*. 82, pp.976–986.
Sah, P., Leu, S.T., Cross, P.C., Hudson, P.J., Bansal, S., 2017. Unraveling the disease consequences and mechanisms of modular structure in animal social networks. *Proceedings of the National Academy of Sciences of the United States of America*. 114, pp.4165–4170.
Sakamoto, H., Kageyama, D., Hoshizaki, S., Ishikawa, Y., 2007. Sex-specific death in the Asian corn borer moth (*Ostrinia furnacalis*) infected with Wolbachia occurs across larval development. *Genome.* 50, pp.645–652.

Salathé, M., Jones, J.H., 2010. Dynamics and Control of Diseases in Networks with Community Structure. *PLOS Computational Biology*. 6, e1000736.
Saltz, J.B., 2011. NATURAL GENETIC VARIATION IN SOCIAL ENVIRONMENT CHOICE: CONTEXT-DEPENDENT GENE-ENVIRONMENT CORRELATION IN *DROSOPHILA MELANOGASTER*. *Evolution* 65, pp.2325–2334.
Sanchez, A., Devevey, G., Bize, P., 2011. Female-biased infection and transmission of the gastrointestinal nematode *Trichuris arvicolae* infecting the common vole, *Microtus arvalis*. *International Journal for Parasitology*. 41, pp.1397–1402.
Satterfield, D.A., Maerz, J.C., Altizer, S., 2015. Loss of migratory behaviour increases infection risk for a butterfly host. *Proceedings Biological Sciences*. 282, pp.20141734–20141734.
Sawyer, L.A., Hennessy, J.M., Peixoto, A.A., Rosato, E., Parkinson, H., Costa, R., Kyriacou, C.P., 1997. Natural Variation in a *Drosophila* Clock Gene and Temperature Compensation. *Science*. 278, pp.2117–2120.
Schaller, M., Park, J.H., 2011. The Behavioral Immune System (and Why It Matters). *Current Directions in Psychological Science*. 20, pp.99–103.
Schiffer, J.T., Mayer, B.T., Fong, Y., Swan, D.A., Wald, A., 2014. Herpes simplex virus-2 transmission probability estimates based on quantity of viral shedding. *Journal of The Royal Society Interface*. 11, pp.20140160–20140160.
Schmid-Hempel, P., Schmid-Hempel, R., 1993. Transmission of a pathogen in *Bombus terrestris*, with a note on division of labour in social insects. *Behav Ecol Sociobiol.* 33, pp.319–327.
Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of Image Analysis. *Nat Methods*. 9, pp.671–675.
Schneider, D.S., Ayres, J.S., 2008. Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases. *Nature Reviews Immunology.* 8, pp.889–895.
Schneider, J., Dickinson, M.H., Levine, J.D., 2012. Social structures depend on innate determinants and chemosensory processing in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*.109, pp.17174–17179.
Schradin, C., 2013. Intraspecific variation in social organization by genetic variation, developmental plasticity, social flexibility or entirely extrinsic factors. *Philos Trans R Soc Lond B Biol Sci*. 368.
Schulenburg, H., Ewbank, J.J., 2007. The genetics of pathogen avoidance in *Caenorhabditis elegans*. *Molecular microbiology*. 66, pp.563–70.
Sears, B.F., Rohr, J.R., Allen, J.E., Martin, L.B., 2011. The economy of inflammation: when is less more? *Trends in Parasitology*. 27, pp.382–387.
Seeman, O.D., Nahrung, H.F., 2004. FEMALE BIASED PARASITISM AND THE IMPORTANCE OF HOST GENERATION OVERLAP IN A SEXUALLY TRANSMITTED PARASITE OF BEETLES. *para.* 90, pp.114–118.
Seivwright, L.J., Redpath, S.M., Mougeot, F., Leckie, F., Hudson, P.J., 2005. Interactions between intrinsic and extrinsic mechanisms in a cyclic species: testosterone increases parasite infection in red grouse. *Proc. Biol. Sci.* 272, pp.2299–2304.
Shang, Y., Feng, P., Wang, C., 2015. Fungi That Infect Insects: Altering Host Behavior and Beyond. *PLOS Pathogens.* 11, e1005037.
Shaw, D.J., Dobson, A.P., 1995. Patterns of macroparasite abundance and aggregation in wildlife populations: a quantitative review. *Parasitology.* 111, S111–S133.
Shaw, P.J., Cirelli, C., Greenspan, R.J., Tononi, G., 2000. Correlates of sleep and waking in *Drosophila melanogaster*. *Science*. 287, pp.1834–7.
Shen, Z., Ning, F., Zhou, W., He, X., Lin, C., Chin, D.P., Zhu, Z., Schuchat, A., 2004. Superspreading SARS Events, Beijing, 2003. *Emerging Infectious Diseases*. 10, pp.256–260.
Sheridan, L.A.D., Poulin, R., Ward, D.F., Zuk, M., 2000. Sex differences in parasitic infections among arthropod hosts: is there a male bias? *Oikos.* 88, pp.327–334.

Shi, M., Lin, X.-D., Tian, J.-H., Chen, L.-J., Chen, X., Li, C.-X., Qin, X.-C., Li, J., Cao, J.-P., Eden, J.-S., Buchmann, J., Wang, W., Xu, J., Holmes, E.C., Zhang, Y.-Z., 2016. Redefining the invertebrate RNA virosphere. *Nature.* 540, pp.539–543.
Short, S.M., Lazzaro, B.P., 2010. Female and male genetic contributions to post-mating immune defence in female Drosophila melanogaster. *Proceedings. Biological sciences / The Royal Society*. 277, pp.3649–57.
Short, S.M., Wolfner, M.F., Lazzaro, B.P., 2012. Female *Drosophila melanogaster* suffer reduced defense against infection due to seminal fluid components. *Journal of insect physiology*. 58, pp.1192–201.
Shorter, J., Couch, C., Huang, W., Carbone, M.A., Peiffer, J., Anholt, R.R.H., Mackay, T.F.C., 2015. Genetic architecture of natural variation in Drosophila melanogaster aggressive behavior. *Proceedings of the National Academy of Sciences of the United States of America.* 112, E3555-63.
Shpigler, H.Y., Saul, M.C., Corona, F., Block, L., Cash Ahmed, A., Zhao, S.D., Robinson, G.E., 2017. Deep evolutionary conservation of autism-related genes. *Proceedings of the National Academy of Sciences of the United States of America*. 114, pp.9653–9658.
Silk, M.J., Weber, N.L., Steward, L.C., Hodgson, D.J., Boots, M., Croft, D.P., Delahay, R.J., McDonald, R.A., 2018. Contact networks structured by sex underpin sex-specific epidemiology of infection. *Ecology Letters*. 21, pp.309–318.
Simon, A.F., Chou, M.-T., Salazar, E.D., Nicholson, T., Saini, N., Metchev, S., Krantz, D.E., 2012. A simple assay to study social behavior in *Drosophila*: measurement of social space within a group. *Genes, brain, and behavior*. 11, pp.243–52.
Siva-Jothy, J.A., Monteith, K.M., Vale, P.F., 2018a. Navigating infection risk during oviposition and cannibalistic foraging in a holometabolous insect. *Behav Ecol.* 29 (6) pp.1426 -1435
Siva-Jothy, J.A., Prakash, A., Vasanthakrishnan, R.B., Monteith, K.M., Vale, P.F., 2018b. Oral Bacterial Infection and Shedding in *Drosophila melanogaster*. *Journal of Visualized Experiments.* (135): e57676–e57676.
Siva-Jothy, J.A., Vale, P.F., 2019. Viral infection causes sex-specific changes in fruit fly social aggregation behaviour. *bioRxiv* 630913.
Slawson, J.B., Kim, E., Griffith, L.C., 2009. High-Resolution Video Tracking of Locomotion in Adult *Drosophila Melanogaster*. *Journal of Visualized Experiments* e1096–e1096.
Sokolowski, M B, 2001. *Drosophila*: genetics meets behaviour. *Nature reviews Genetics*. 2, pp.879–90.
Sokolowski, M.B., 1980. Foraging strategies of *Drosophila melanogaster*: A chromosomal analysis. *Behavior Genetics.* 10, pp.291–302.
Stein, R.A., 2011. Super-spreaders in infectious diseases. *International journal of infectious diseases*. 15, e510-3.
Stensmyr, M.C., Dweck, H.K.M., Farhan, A., Ibba, I., Strutz, A., Mukunda, L., Linz, J., Grabe, V., Steck, K., Lavista-Llanos, S., Wicher, D., Sachse, S., Knaden, M., Becher, P.G., Seki, Y., Hansson, B.S., 2012. A Conserved Dedicated Olfactory Circuit for Detecting Harmful Microbes in *Drosophila*. *Cell.* 151, 1345–1357.
Stevanovic, A., Johnson, K.N., 2015. Infectivity of *Drosophila* C virus following oral delivery in *Drosophila* larvae. *J. Gen. Virol.* 96(6). pp.1490-6
Strauss, R., 2002. The central complex and the genetic dissection of locomotor behaviour. *Current Opinion in Neurobiology*. 12, pp.633–638.
Strobl, C., Boulesteix, A.-L., Zeileis, A., Hothorn, T., 2007. Bias in random forest variable importance measures: illustrations, sources and a solution. *BMC Bioinformatics*. 8, 25.
Strobl, C., Hothorn, T., Zeileis, A., 2009. Party on! A new, conditional variable-importance measure for random forests available in the party package. *The R Journal*. 1, pp.14–17.
Sullivan, K., Fairn, E., Adamo, S.A., 2016. Sickness behaviour in the cricket *Gryllus texensis*: Comparison with animals across phyla. *Behavioural processes*. 128, pp.134–143.

Surendran, S., H?ckesfeld, S., W?schle, B., Pankratz, M.J., 2017. Pathogen-induced food evasion behavior in *Drosophila* larvae. *The Journal of Experimental Biology*. 220, pp.1774–1780.
Susi, H., Barrès, B., Vale, P.F., Laine, A.-L., 2015a. Co-infection alters population dynamics of infectious disease. *Nature Communications*. 6, 5975.
Susi, H., Vale, P.F., Laine, A.-L., 2015b. Host Genotype and Coinfection Modify the Relationship of within and between Host Transmission. *The American Naturalist* 186 pp.252-263.
Tavalire, H.F., Blouin, M.S., Steinauer, M.L., 2016. Genotypic variation in host response to infection affects parasite reproductive rate. *International Journal for Parasitology*. 46, pp.123–131.
Taylor, K., Kimbrell, D., 2007. Host Immune Response and Differential Survival of the Sexes in *Drosophila*. *Fly*. 1, pp.197–204.
Thomas-Orillard, M., 1984. Modifications of Mean Ovariole Number, Fresh Weight of Adult Females and Developmental Time in *Drosophila melanogaster* Induced by *Drosophila* C Virus. *Genetics.* 107, pp.635–644.
Thompson, O., Gipson, S.A.Y., Hall, M.D., 2017. The impact of host sex on the outcome of co-infection. *Scientific Reports*. 7, 910.
Thrall, P.H., Antonovics, J., Dobson, A.P., 2000. Sexually transmitted diseases in polygynous mating systems: prevalence and impact on reproductive success. *Proc Biol Sci.* 267, pp.1555–1563.
Tinsley, M.C., Blanford, S., Jiggins, F.M., 2006. Genetic variation in *Drosophila melanogaster* pathogen susceptibility. *Parasitology*. 132, pp.767–773.
Tizard, I.R., 2009. Veterinary immunology: an introduction, 8th ed. ed. *St Louis, Mo. : Saunders Elsevier.*
Tjørnløv, R.S., Kissling, W.D., Barnagaud, J.-Y., Bøcher, P.K., Høye, T.T., 2015. Oviposition site selection of an endangered butterfly at local spatial scales. *J Insect Conserv*. 19, pp.377–391.
Tolwinski, N.S., 2017. Introduction: *Drosophila*—A Model System for Developmental Biology. *J Dev Biol* 5.
Trannoy, S., Chowdhury, B., Kravitz, E.A., 2015. Handling alters aggression and "loser" effect formation in *Drosophila melanogaster*. Learning & Memory 22, pp.64–68.
Ueda, A., Kidokoro, Y., 2002. Aggressive behaviours of female *Drosophila melanogaster* are influenced by their social experience and food resources. *Physiological Entomology*. 27, pp.21–28.
Unckless, R.L., 2011. A DNA Virus of Drosophila. *PLOS ONE*. 6, e26564.
Vale P. F., Choisy M., Little T. J., 2013. Host nutrition alters the variance in parasite transmission potential. *Biology Letters*. 9, 20121145.
Vale, P.F., Choisy, M., Little, T.J., 2013. Host nutrition alters the variance in parasite transmission potential. *Biol Lett.* 9.
Vale, P.F., Fenton, A., Brown, S.P., 2014. Limiting Damage during Infection: Lessons from Infection Tolerance for Novel Therapeutics. *PLoS Biology* 12.
Vale, P.F., Jardine, M.D., 2016. Infection avoidance behavior: Viral exposure reduces the motivation to forage in female *Drosophila melanogaster*. *Fly.* pp.1–7.
Vale, P.F., Jardine, M.D., 2015. Sex-specific behavioural symptoms of viral gut infection and Wolbachia in *Drosophila melanogaster*. *Journal of Insect Physiology*. 82, pp.28–32.
van Lunzen, J., Altfeld, M., 2014. Sex Differences in Infectious Diseases–Common but Neglected. *J Infect Dis.* 209, pp.79–80.
Vander Waal, E.K., Yip, H., McLoughlin, P.D., 2012. Sex-based differences in density-dependent sociality: an experiment with a gregarious ungulate. *Ecology*. 93, pp.206–212.
VanderWaal, K.L., Ezenwa, V.O., 2016. Heterogeneity in pathogen transmission: mechanisms and methodology. *Functional Ecology*.
Vasconcelos, S.D., 1996. Alternative Routes for the Horizontal Transmission of a Nucleopolyhedrovirus. *Journal of Invertebrate Pathology*. 68, pp.269–274.

Vazquez-Prokopec, G.M., Perkins, T.A., Waller, L.A., Lloyd, A.L., Reiner, R.C., Scott, T.W., Kitron, U., 2016. Coupled Heterogeneities and Their Impact on Parasite Transmission and Control. *Trends in Parasitology.* 32, pp.356–367.
Vijendravarma, R.K., Narasimha, S., Kawecki, T.J., 2013. Predatory cannibalism in Drosophila melanogaster larvae. *Nature Communications.* 4, 1789.
Vincent, C.M., Sharp, N.P., 2014. Sexual antagonism for resistance and tolerance to infection in Drosophila melanogaster. *Proceedings Biological sciences / The Royal Society*. 281, pp.20140987–20140987.
Wallace, R., Wallace, R.G., 2015. Blowback: new formal perspectives on agriculturally driven pathogen evolution and spread. *Epidemiology & Infection*. 143, pp.2068–2080.
Walum, H., Westberg, L., Henningsson, S., Neiderhiser, J.M., Reiss, D., Igl, W., Ganiban, J.M., Spotts, E.L., Pedersen, N.L., Eriksson, E., Lichtenstein, P., 2008. Genetic variation in the vasopressin receptor 1a gene (*AVPR1A*) associates with pair-bonding behavior in humans. *Proceedings of the National Academy of Sciences of the United States of America*.105, pp.14153–14156.
Wang, J.B., Lu, H.-L., St. Leger, R.J., He, Y., Carlson, J., Tsang, G., Evans-Holm, M., Hiesinger, P., Schulze, K., Rubin, G., Hoskins, R., 2017. The genetic basis for variation in resistance to infection in the *Drosophila melanogaster* genetic reference panel. *PLOS Pathogens.* 13, e1006260–e1006260.
Wayland, M.T., Defaye, A., Rocha, J., Jayaram, S.A., Royet, J., Miguel-Aliaga, I., Leulier, F., Cognigni, P., 2014. Spotting the differences: Probing host/microbiota interactions with a dedicated software tool for the analysis of faecal outputs in *Drosophila*. *Journal of Insect Physiology*. 69, pp.126–135.
Weary, D.M., Huzzey, J.M., von Keyserlingk, M. a. G., 2009. BOARD-INVITED REVIEW: Using behavior to predict and identify ill health in animals. *J Anim Sci*. 87, pp.770–777.
Webster, C.L., Waldron, F.M., Robertson, S., Crowson, D., Ferrari, G., Quintana, J.F., Brouqui, J.M., Bayne, E.H., Longdon, B., Buck, A.H., Lazzaro, B.P., Akorli, J., Haddrill, P.R., Obbard, D.J., 2015. The Discovery, Distribution, and Evolution of Viruses Associated with *Drosophila melanogaster*. *PLoS Biol*. 13, e1002210–e1002210.
Weissbrod, A., Shapiro, A., Vasserman, G., Edry, L., Dayan, M., Yitzhaky, A., Hertzberg, L., Feinerman, O., Kimchi, T., 2013. Automated long-term tracking and social behavioural phenotyping of animal colonies within a semi-natural environment. *Nature Communications*. 4, 2018.
White, L.A., Forester, J.D., Craft, M.E., 2018. Covariation between the physiological and behavioral components of pathogen transmission: host heterogeneity determines epidemic outcomes. *Oikos.* 127, pp.538–552.
White, L.A., Forester, J.D., Craft, M.E., 2017. Using contact networks to explore mechanisms of parasite transmission in wildlife. *Biological Reviews*. 92, pp.389–409.
Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. *Springer-Verlag New York.*
Wilfert, L., Long, G., Leggett, H.C., Schmid-Hempel, P., Butlin, R., Martin, S.J.M., Boots, M., 2016. Deformed wing virus is a recent global epidemic in honeybees driven by Varroa mites. *Science.* 351, pp.594–597.
Williams, K.J., Ward, M.P., Dhungyel, O.P., Hall, E.J.S., Van Breda, L., 2014. A longitudinal study of the prevalence and super-shedding of *Escherichia coli* O157 in dairy heifers. *Veterinary Microbiology*. 173, pp.101–109.
Williams, T., Hernández, O., 2006. Costs of cannibalism in the presence of an iridovirus pathogen of Spodoptera frugiperda. *Ecological Entomology*. 31, pp.106–113.
Wilson, K., Bjørnstad, O.N., Dobson, A.P., Merler, S., Poglayen, G., Read, A.F., Skorping, A., 2002. Chapter 2: Heterogeneities in macroparasite infections: patterns and processes. The ecology of wildlife diseases. *Oxford University Press, Oxford*. pp.6–44.

Winslow, J.T., Hastings, N., Carter, C.S., Harbaugh, C.R., Insel, T.R., 1993. A role for central vasopressin in pair bonding in monogamous prairie voles. *Nature.* 365, 545.
Woolhouse, M.E.J., Dye, C., Etard, J.-F., Smith, T., Charlwood, J.D., Garnett, G.P., Hagan, P., Hii, J.L.K., Ndhlovu, P.D., Quinnell, R.J., Watts, C.H., Chandiwana, S.K., Anderson, R.M., 1997. Heterogeneities in the transmission of infectious agents. *Proceedings of the National Academy of Sciences of the United States of America.* 94, pp.338–342.
Woyciechowski, M., Kozłowski, J., 1998. Division of labor by division of risk according to worker life expectancy in the honey bee (*Apis mellifera L.*). *Apidologie*. 29, pp.191–205.
Zhong, W., McClure, C.D., Evans, C.R., Mlynski, D.T., Immonen, E., Ritchie, M.G., Priest, N.K., 2013. Immune anticipation of mating in *Drosophila*: *Turandot M* promotes immunity against sexually transmitted fungal infections. *Proceedings. Biological sciences / The Royal Society*. 280, pp.20132018–20132018.
Zhu, Y., Chen, H., Fan, Jinghua, Wang, Y., Li, Y., Chen, J., Fan, JinXiang, Yang, S., Hu, L., Leung, H., Mew, T.W., Teng, P.S., Wang, Z., Mundt, C.C., 2000. Genetic diversity and disease control in rice. *Nature.* 406, 718.
Zuk, M., McKean, K.A., 1996. Sex differences in parasite infections: Patterns and processes. *International Journal for Parasitology*. 26, pp.1009–1024.

Appendices

7.1 Chapter 2

7.1.1 Supplementary Figures

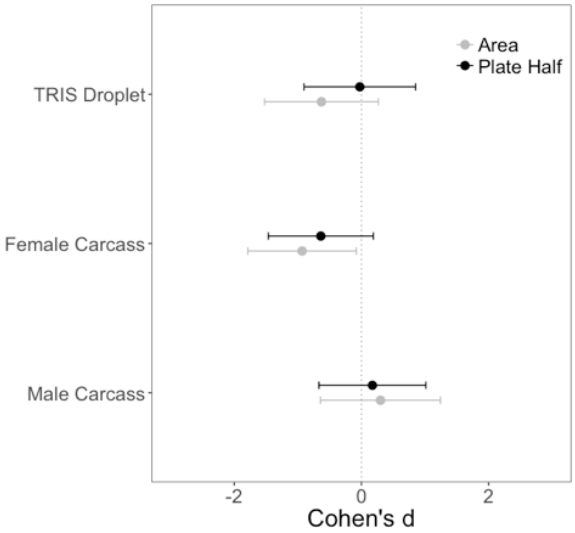


Figure S1. Larval foraging choice. Distribution of effect sizes for relationships observed between infected and non-infected food sources when larval foraging avoidance was measured according to plate area (grey) and half (black). Plots show Cohen's d (± 95% confidence interval).

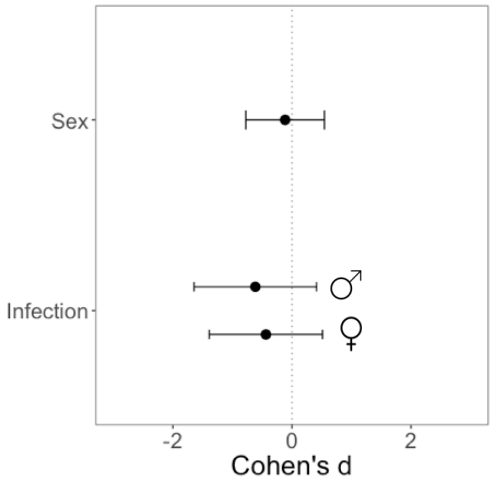


Figure S2. Pupal eclosion rates. Distribution of effect sizes for relationships observed between males and females according to carcass infection and sex on pupal eclosion rates. Plots show Cohen's d (± 95% confidence interval).

7.1.2 Publication

Siva-Jothy, J. A., Monteith, K.M., Vale, P. F. (2018) Navigating infection risk during oviposition and foraging in a holometabolous insect. *Behavioral Ecology*. Vol. 29 (6) pp.1426 -1435

Behavioral Ecology

The official journal of the ISBE International Society for Behavioral Ecology

Behavioral Ecology (2018), 29(6), 1426–1435. doi:10.1093/beheco/ary106

Original Article

Navigating infection risk during oviposition and cannibalistic foraging in a holometabolous insect

Jonathon A. Siva-Jothy,[a,*,•] Katy M. Monteith,[a,*] and Pedro F. Vale[a,b,•]

[a]Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, EH9 3FL Edinburgh, UK and [b]Centre for Immunity, Infection and Evolution, University of Edinburgh, EH9 3FL Edinburgh, UK

Received 28 March 2018; revised 20 June 2018; editorial decision 5 July 2018; accepted 01 August 2018; Advance Access publication 9 August 2018.

Deciding where to eat and raise offspring carries important fitness consequences for all animals, especially if foraging, feeding, and reproduction increase pathogen exposure. In insects with complete metamorphosis, foraging mainly occurs during the larval stage, while oviposition decisions are made by adult females. Selection for infection avoidance behaviors may therefore be developmentally uncoupled. Using a combination of experimental infections and behavioral choice assays, we tested if *Drosophila melanogaster* fruit flies avoid infectious environments at distinct developmental stages. When given conspecific fly carcasses as a food source, larvae did not discriminate between carcasses that were clean or infected with the pathogenic Drosophila C Virus (DCV), even though cannibalism was a viable route of DCV transmission. When laying eggs, DCV-infected females did not discriminate between infectious and noninfectious carcasses, and laying eggs near potentially infectious carcasses was always preferred to sites containing only fly food. Healthy mothers, however, laid more eggs near a clean rather than an infectious carcass. Avoidance during oviposition changed over time: after an initial oviposition period, healthy mothers stopped avoiding infectious carcasses. We interpret this result as a possible trade-off between managing infection risk and maximizing reproduction. Our findings suggest infection avoidance contributes to how mothers provision their offspring and underline the need to consider infection avoidance behaviors at multiple life-stages.

Key words: ***Drosophila*, Drosophila C virus, foraging, infection avoidance, infection risk, oviposition site choice.**

INTRODUCTION

Behavioral immunity, the suite of behaviors that allow animals to avoid contact with infectious environments or conspecifics, is the first line of defense against infection (Parker *et al.* 2011; Schaller and Park 2011; Curtis 2014). Avoidance of infection relies on detecting cues of parasite presence—such as visual cues of infection risk or secondary pathogen metabolites—and integrating this sensory information to avoid sources of infection (Kiesecker *et al.* 1999; Kavaliers *et al.* 2004; Stensmyr *et al.* 2012; Kacsoh *et al.* 2013; Babin *et al.* 2014; Meisel *et al.* 2014; Kurz *et al.* 2017). In addition to external cues of infection risk, the internal state of the animal, including its physiological status as a result of prior pathogen exposure, may also affect the ability to detect and avoid infection (Curtis *et al.* 2011; Klemme and Karvonen 2016; Vale and Jardine 2017).

Avoiding contact with pathogens allows healthy individuals to escape the pathology that results from infection, and also prevents the deployment of the immune response, which may be metabolically costly and even cause immunopathology (Schaller and Park 2011; Sears *et al.* 2011; Curtis 2014). Despite these clear advantages, avoiding infection completely is rarely possible. Foraging and feeding, for example, are vital aspects of host ecology, and are key to organismal reproduction and fitness, but they are also major routes of pathogen transmission (Hall *et al.* 2007; Lefèvre and Roode *et al.* 2012).

Foraging and feeding are particularly important for holometabolous insect larvae, which devote most of their time to these behaviors. In situations of severe nutritional scarcity, larvae may even resort to cannibalism. For example, larvae of the fruit fly *Drosophila melanogaster* readily eat the carcasses of conspecifics following periods of starvation (Vijendravarma *et al.* 2013; Ahmad *et al.* 2015). Cannibalism may appear to be a beneficial strategy when the alternative is starvation but may increase the risk of trophic transmission of pathogens and parasites, especially if infected individuals are more likely to be targeted for cannibalism. While larvae of many insect species are frequently observed to avoid infectious environments or food sources (de Roode and Lefèvre 2012), it is

Address correspondence to J.A. Siva-Jothy. E-mail: s1115177@ed.ac.uk.
*These authors contributed equally to this work, and would like to share first authorship.

currently unclear if trophic infection avoidance occurs during cannibalistic scavenging.

Beyond foraging during the larval stage, choosing where to oviposit or rear offspring is another important life-history decision, but can be risky if individuals are unable to identify and avoid potentially infectious environments. The environment in which adult insects choose to oviposit is therefore a major determinant both of offspring environmental quality and infection risk (Lefèvre and Roode *et al.* 2012; Lefèvre *et al.* 2012; Kacsoh *et al.* 2013). Infection avoidance by insects during oviposition has been observed in response to a number of parasites and appears to be driven by diverse sensory cues, including avoidance of parasitoid wasp visual cues (Kacsoh *et al.* 2013), and olfactory detection of bacteria and fungi (Stensmyr *et al.* 2012; Kurz *et al.* 2017). Together, both adult oviposition choice and larval food preference determine the likelihood of infection in the early life-stages of holometabolous insects, and therefore both behaviors play an important role in disease transmission dynamics (Kiesecker *et al.* 1999; Ezenwa *et al.* 2016).

Here, we investigate larval foraging and adult oviposition in a holometabolous insect—the fruit fly *Drosophila melanogaster*—in the context of infection avoidance. Our study consisted of choice assays performed on either larval or adult stage *D. melanogaster*. Fly larvae were presented with a choice of scavenging on either a clean, noninfectious adult fly carcass, or a carcass that had been previously inoculated with a systemic Drosophila C Virus (DCV) infection (Figure 1a). In a second experiment, we tested adult oviposition choice by giving female flies the choice to lay eggs on a clean food source, a clean food source also containing a clean carcass, and a food source containing a carcass with a systemic DCV infection (Figure 1b). This 3-way choice assay allowed us to examine an important conflict faced by mothers: a carcass may present an additional nutritional source for future offspring but may also present a potential risk of infection. In both experiments, we assessed the fitness consequences of choices at both life-stages by following the development of larvae or laid eggs.

MATERIALS AND METHODS

Fly lines and rearing conditions

In both experiments, we used laboratory stocks of *D. melanogaster* Oregon R (OreR). We kept fly stocks in plastic bottles (6oz; Genesee Scientific, San Diego, CA) on a standard diet of Lewis medium (Lewis 2014) at 18 ± 1 °C with a 12-h light:dark cycle. Stocks were tipped approximately every 21 days into new bottles. Before the experiments, we transferred flies to clean bottles and maintained them at low density (~50 flies per bottle) for a minimum of 2 generations at 25 ± 1 °C with a 12-h light:dark cycle.

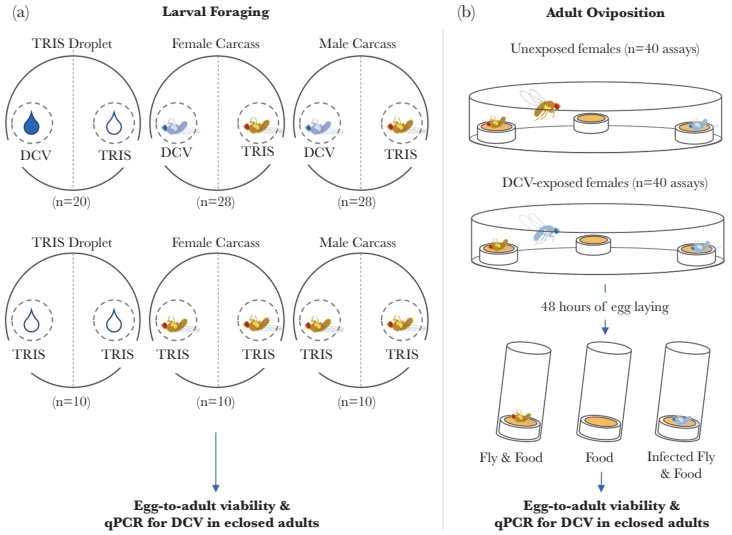


Figure 1
Experimental design. (a) Two-choice chamber used to measure larval foraging choice when presented with infectious and noninfectious food sources and the life-history data collected 24 h after the 72-h assay. Petri dishes were set up as either 2-choice plates (containing a DCV infectious and noninfectious TRIS droplet or food source) or control plates (containing only noninfectious TRIS droplets or food sources). Eggs were placed at the center of each plate, allowed to hatch and left for 72 h whereupon the position of larvae was recorded to assay infection avoidance. (b) Three-choice chamber used to assay oviposition site choice in infected and uninfected mothers when presented with 3 sites containing just food, food and a fly carcass, and food and an infected fly carcass. The number of eggs laid at each site was measured twice at two 24-h intervals. After 48 h, oviposition sites were removed and clutches were allowed to develop to adults whereupon the viral load of a randomly selected subsample was assayed.

Virus culture and infection

DCV is a horizontally transmitted positive-sense ssRNA virus of the Dicistroviridae family (Huszar and Imler 2008). DCV infection establishes in the digestive, reproductive and fat tissues, resulting in a range of behavioral and physiological pathologies in both larval and adult stage flies, including reduced locomotor activity, metabolic and reproductive dysfunction, and eventually death (Arnold *et al.* 2013; Chtarbanova *et al.* 2014; Stevanovic and Johnson 2015; Vale and Jardine 2015; Gupta *et al.* 2017). The DCV isolate used in this experiment was originally isolated in Charolles, France (Jousset *et al.* 1977) and was grown in Schneider Drosophila Line 2 (DL2) as previously described (Vale and Jardine 2015), serially diluted ten-fold in TRIS-HCl solution (pH = 7.3), aliquoted and frozen at −80 °C until required. To infect flies, we bent Austerlitz insect pins (0.15 mm in diameter) at a 90° angle ~0.5 mm from the tip, dipped the tip in DCV, and inserted it into the intersegmental membrane under the fly's wing, with the fly under CO_2 anesthesia. Control infections employed the same protocol but with a needle tip dipped in sterile TRIS solution.

Infection avoidance during larval foraging

We had previously observed that fly larva would readily cannibalize dead adult fly carcasses (Supplementary Video S1), and we hypothesized that cannibalism could be viable route of transmission. We would therefore expect selection for the avoidance of potentially infected carcasses, and so we tested if healthy fly larvae could discriminate between healthy and potentially infectious fly carcasses. To generate these carcasses, we randomly selected 4–7-day-old male and female flies from an age-matched population. For each sex, we stabbed half of the flies with DCV 10^7 DCV Infectious Units (IU)/mL and the other half stabbed with sterile TRIS buffer. Following 6 days (to allow viral replication), we froze live flies at −80 °C until required. We confirmed the infection status of the carcasses using DCV-specific quantitative reverse transcription PCR (RT-qPCR) (see below) by randomly picking 5 male and 5 female flies.

We carried out a 2-choice assay by placing ~100 fly eggs at the center of each Petri dish containing ~20 mL solid agar (5% sugar) and allowed the resulting 3rd instar larvae to forage towards either a clean fly carcass or a carcass infected with DCV, placed at an equidistant positon from the eggs (3 cm) (Figure 1a). Eggs were collected from apple-agar plates placed in a population cage containing approximately 1500 adult flies for 24 h. Eggs were suspended in Ringer's solution and then pipetted as 10 µL squirts onto the agar plates. We set up 56 "choice" assays where healthy larvae could choose between a clean or DCV-infected carcass, and 20 "control" assays, where both carcasses were clean (half of assays contained male carcasses, and the other half contained female carcasses). Infection avoidance was analyzed by comparing the preference or larvae when given a choice between infected and clean carcass (choice plate) to the preference when both carcasses are clean (control plates). To differentiate between any effects of carcass degradation from a direct effect of DCV presence on healthy larval choice, we also set up an additional 30 plates without fly carcasses, containing 10 µL of DCV (10^7DCV IU/mL) and 10 µL of TRIS (2-choice; $N = 20$) or only TRIS (control; $N = 10$). Eighteen of the 106 plates set up across all treatments were excluded from the final dataset due to damage to the surface of the agar which could have affected larval movement. We conducted all assays at 25 ± 1 °C with a 12-h light:dark cycle before being photographed after 72 h. We marked images using Adobe Photoshop CS3 to count the number of larvae within each plate half and within an area immediately surrounding the carcasses/droplets (~2.2 cm in diameter—see Figure 1a).

Larval infection status and virus quantification

After 96 h, we randomly selected 10 larvae found on or within the closest proximity to each carcass in 20 "choice plates" and one carcass in 6 "control plates" to assess DCV infection status and quantify viral load in these pooled groups of 10 larvae. We performed viral quantification using absolute quantification of DCV RNA copies using qRT-PCR. Total RNA was extracted by homogenizing the flies or larvae in TRI Reagent (Invitrogen, Carlsbad, CA) and using Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA), including a DNase step. The eluted RNA was then reverse-transcribed with M-MLV reverse transcriptase (Promega, Madison, WI) and random hexamer primers, and then diluted 1:1 with nuclease free water. The qRT-PCR was performed on an Applied Biosystems StepOnePlus system using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA) using the following forward and reverse primers, which include 5′-AT rich flaps to improve fluorescence (Afonina *et al.* 2007) (DCV_Forward: 5′ AATAAATCATAAGCCACTGTGATTGATACAACAGAC 3′; DCV_Reverse: 5′ AATAAATCATAAGAAGCACGATAC TTCTTCCAAACC 3′; with the following PCR cycle: 95 °C for 2 min followed by 40 cycles of: 95 °C for 10 s followed by 60 °C for 30 s. Two qRT-PCR reactions (technical replicates) were carried out per sample. For absolute quantification of DCV, the concentrations of DCV in the samples were extrapolated from a standard curve created from a 10-fold serial dilution ($1–10^{-6}$) of DCV cDNA. We considered any amplification obtained above a Ct-value of 36 to be a false positive and took them as zero-values during statistical analysis. This Ct cut-off was chosen because it corresponds to the theoretical limit of detection of a single DNA copy given a reaction efficiency close to 100% (Caraguel et al. 2011), and is supported by our standard curves where Ct 36 corresponded to 1-10 DCV copies. Furthermore, several of our samples with Ct>36 presented melt curves with multiple peaks indicating a limit for accurate detection of DCV.

Larval development and infection status

To analyze the effect of foraging choice on larval development, after 96 h we removed 15 larvae found on or within the closest proximity to each carcass from 20 "choice" plates and from one carcass on 6 "control" plates. We transferred larvae from each carcass together into plastic vials containing Lewis medium and recorded the number of larvae that developed into pupae and the number of eclosed adults. We froze a subset of these adults in TRI reagent and tested their infection status to verify DCV infection's persistence through metamorphosis

Infection avoidance during oviposition

Following our test of infection avoidance at the larval stage, we carried out a second experiment to test the oviposition preference of female *D. melanogaster* when presented with a choice of clean and potentially infectious oviposition sites. We made choice chambers by joining 2 bases of transparent plastic Petri dishes with adhesive tape, making a chamber 10 cm in diameter and 2 cm in height. Chambers contained 3 oviposition sites comprised of upturned caps filled with Lewis medium, arranged in a triangle, each site, 50 mm from the other two (Figure 1b). Oviposition sites contained either only Lewis medium, Lewis medium and an uninfected female fly carcass, or Lewis medium and a DCV-infected female fly carcass (infection protocol described above). Fly carcasses were

obtained using the protocol described above but with an infectious dose of 10^8 DCV IU/mL.

Three-day-old male and female flies were isolated as virgins and females were stabbed with either a virus-contaminated (10^8 DCV IU/mL) or sterile, virus-free control solution. Following infection, females to be used in the oviposition assay were introduced to 2 uninfected males for mating for 72 h. After which a single mated female was introduced to an oviposition chamber and placed at 25 °C (12-h light:dark cycle) to await oviposition. Two females (1 infected and 1 uninfected) laid no eggs during the experiment so were excluded from the final dataset. In total, we measured the oviposition choice of 80 females. As DCV has been reported to affect *D. melanogaster* fecundity (Thomas-Orillard 1984; Gomariz-Zilber and Thomas-Orillard 1993; Gupta *et al.* 2017), we measure infection avoidance during oviposition using the number, rather than proportion, of eggs laid at a particular site. To count the number of eggs laid on each oviposition site, we took photos of individual oviposition sites with a Leica MC170 HD camera attachment on a Leica 0.32×/WD 200 mm S8APO microscope (Leica microsystems, Wetzlar, Germany) after females had been in the chambers for 24 and 48 h.

Fitness consequences of oviposition site choice

We quantified the potential fitness consequences of oviposition preference by transferring all oviposition sites, including carcass (if present), to individual vials and recorded egg-to-adult viability. We pooled adults that eclosed from clutches during this experiment together in TRI reagent and analyzed DCV infection using the same protocol as above. A total of 24 clutches were analyzed in this way; we excluded 6 of these due to degradation or contamination during qPCR preparation.

Statistical analyses

In the larval choice experiment, we analyzed the proportion of larvae choosing a given plate half or carcass area; larval DCV titers; the proportion of larvae developing into pupae (logit transformed); and the proportion of pupae that developed into adult flies (logit transformed) and adult DCV titres. All response variables, except adult DCV titres, were analyzed using Generalized Linear Models (GLMs) with "carcass sex" and "carcass infection status" and their interactions as fixed effects. Adult DCV titres in flies originally collected from an uninfected or infected carcasses were compared using a Mann–Whitney *U* test. In the adult oviposition experiment, we used the number of eggs laid at each oviposition site to assess infection avoidance. We analyzed egg counts, rather than the proportion of eggs laid on each oviposition site, to account for potential differences in fecundity between infected and uninfected flies (Thomas-Orillard 1984; Gomariz-Zilber and Thomas-Orillard 1993; Gupta *et al.* 2017). The number of eggs laid was analyzed using a generalized linear mixed model (GLMM) with Poisson distributed error. Our model used a full factorial 3-way interaction between oviposition site, maternal infection status and the 24-h period eggs were laid. The total number of eggs laid and the choice chamber were included as random effects, with the latter nested within the fly's infection status, to account for repeated measures. The proportion of eggs that later eclosed as adults (egg-to-adult viability) was analyzed using a GLMM with a binomially distributed error, with oviposition site included as a fixed effect. All statistical analyses and graphics were carried out and produced in R 3.3.0 using the *ggplot2* (Wickham *et al.* 2016), *lme4* (Bates *et al.* 2018: 4), and *multcomp* (Hothorn *et al.* 2017) packages.

RESULTS

Larval flies do not avoid infectious food sources when scavenging

Fly larvae that hatched from eggs placed in the center of the Petri dish, dispersed towards and consumed the fly carcasses placed at the edges of the dish (Supplementary Video S1). We found no evidence that fly larvae can avoid infected food sources. Regardless of the measure of preference (plate half larvae were found in or the area surrounding each carcass or TRIS droplet) larvae on choice plates showed no significant preference for clean or infected fly carcasses when compared to larvae on control plates (Figure 2a,b; Table 1). While the borderline significance of this effect could indicate a general trend of larvae avoiding infected carcasses, we found the effect size (Cohen's *d*) to be close to zero with relatively narrow confidence intervals (Supplementary Figure 1).

DCV is transmitted to larvae when scavenging on infected carcasses

DCV was detected in larvae collected from plates containing an infected carcass (Figure 3a, Table 1), confirming that

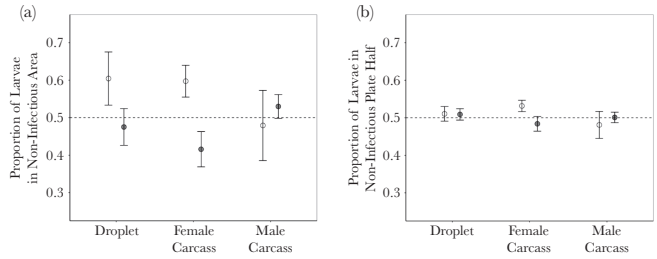


Figure 2
Larval foraging choice. Mean ± SE proportion of larvae on choice plates after 72 h found (a) within area 2.2 cm in diameter of the noninfectious food source and (b) on the noninfectious food source's half of the plate. Results from both choice (white points) and control plates (gray points) are shown. In the case of control plates, where only noninfectious food sources are present, the mean ± SE is derived from the proportion of larvae present at a randomly selected side of the plate. Food sources included droplets of TRIS, a male carcass or female carcass.

Table 1
Model outputs for statistical tests performed on all experiments testing the causes and costs of infection avoidance in *D. melanogaster* larval foraging

Response	Predictor	df	*F*	*P*-value
Larval Foraging Choice by Plate Half	Carcass Sex/TRIS	2	0.599	0.741
	Carcass Infection	1	0.632	0.426
	Carcass Sex/TRIS × Carcass Infection	2	2.76	0.251
Larval Foraging Choice by Carcass Area	Carcass Sex/TRIS	2	0.512	0.774
	Carcass Infection	1	3.60	0.0579
	Carcass Sex/TRIS × Carcass Infection	2	4.50	0.106
Larval DCV Titre	Carcass Sex	1	0.998	0.329
	Carcass Infection	2	5.84	0.0248*
	Carcass Sex × Carcass Infection	2	0.340	0.566
Number of Larvae to Pupate	Carcass Sex	1	13.3	0.0003***
	Carcass Infection	2	0.0745	0.963
	Carcass Sex × Carcass Infection	2	0.618	0.734
Number of Pupae to Eclose	Carcass Sex	1	0.0174	0.895
	Carcass Infection	2	0.180	0.914
	Carcass Sex × Carcass Infection	2	0.149	0.928

Significant predictors are marked with asterisks (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

scavenging infected carcasses is a viable route of virus transmission. As expected, larvae surrounding DCV-infected carcasses were found to have significantly higher DCV titers when compared to larvae collected from control plates (which contained only uninfected carcasses). However, we also detected DCV infection in larvae surrounding clean carcasses that were housed in a 2-choice plate (containing both infected and uninfected carcasses) (Figure 3a), suggesting that some larvae may have moved between food sources in these plates during the assay.

No effect of virus acquisition on larval development

Acquiring infection by scavenging on infectious carcasses had no detectable effect on larval development into pupae (Figure 3b), or in the proportion of pupae that eclosed as adults (Figure 3c; Table 1). However, more larvae developed to pupal stage when they fed on a female carcass (Figure 3b; Table 1): 50% of larvae feeding on female carcasses reached pupation, while a significantly lower proportion (32%) reached pupation if they had fed on male carcasses (Figure 3b). Following pupation, there was no effect of carcass sex or infection status on the proportion of pupae that eclosed as adults (Figure 3c, Table 1; Supplementary Figure 2).

Virus acquired during the larval stage can persist into adulthood

We measured DCV titers in flies that eclosed as adults (Figure 3d). While no DCV infection was detected in flies originally collected near clean carcasses, we detected DCV in 9 out of 15 adult flies that were collected from infected carcasses, suggesting that DCV infection can persist through metamorphosis into the adult insect stage. The amount of virus in flies that were collected from infected carcasses was significantly higher than those collected from uninfected carcasses ($U = 12$, $P = 0.029$, one-tailed).

DCV infection increases fecundity

In addition to measuring DCV avoidance by the number of eggs laid, we measured the total number of eggs laid over the course of the 48 h. Infected mothers laid significantly more eggs than healthy mothers (Figure 4a; Table 2).

Oviposition preference changes over time and depends on the female's infection status

The oviposition sites where mothers laid their eggs changed over time in a manner dependent on the mother's infection status, as indicated by the significant 3-way interaction between time, oviposition site, and the mother's infection status (Figure 4b,c; Table 2). This means that within the first 24-h period, uninfected female flies laid significantly more eggs at sites containing a clean carcass compared to sites with an infected carcass or just food (Figure 4b; pairwise contrasts, $P < 0.001$). Female flies infected with DCV, however, did not distinguish between infected and clean carcasses, but still laid significantly fewer eggs at sites without any carcass (Figure 4b; pairwise contrasts, $P < 0.0001$). In the 24–48-h observation period, uninfected females still laid more eggs at sites with carcasses, but no longer preferred the sites containing a clean carcass (Figure 4c, Table 2; pairwise contrast, $P = 0.99$). DCV-infected females also laid more eggs at sites with an uninfected carcass (pairwise contrast, $P < 0.0001$), but laid even more eggs on sites containing an infected carcass (Figure 4c; pairwise contrast, $P < 0.001$).

Fitness consequences of oviposition preference

Egg-to-adult viability differed significantly between oviposition sites and was lower in food-only sites compared to sites containing a carcass (Figure 5a; Table 2). Clutches emerging at carcass sites however, did not differ in their egg-to-adult viability (Figure 5a; Table 2), even though we detected significantly more DCV in flies that developed around DCV-infected carcasses (Figure 5b). The infection status of mothers did not affect egg-to-adult viability (Figure 5a; Table 2) or on the viral load of these clutches (Figure 5b; Table 2).

DISCUSSION

Viral infection is widespread among invertebrates (Webster *et al.* 2015; Shi *et al.* 2016), and can cause considerable morbidity and mortality (Escobedo-Bonilla *et al.* 2008; Arnold *et al.* 2013; Wilfert *et al.* 2016; Gupta *et al.* 2017). We should therefore expect selection for mechanisms that allow hosts to detect and avoid infectious conspecifics or potentially infectious environments (Kiesecker *et al.* 1999; Curtis 2014). In the present work, we examined how larval

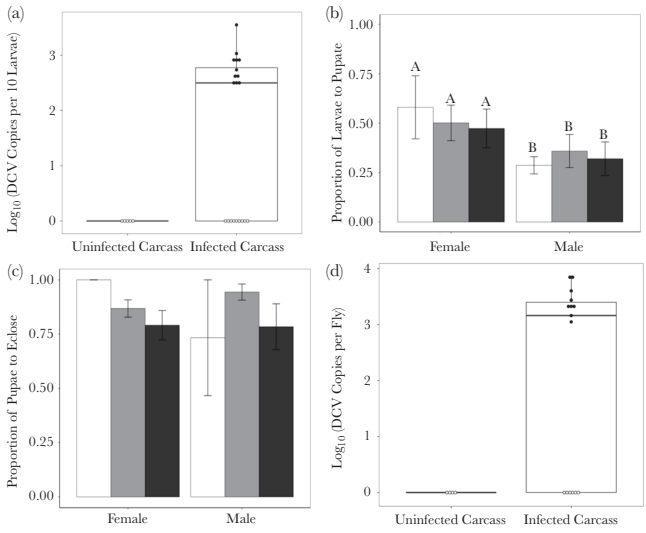


Figure 3
Fitness consequences of infectious scavenging. (a) The number of DCV copies present in larvae, quantified immediately after choice assays having fed on an uninfected carcass on a control plate or a choice plate and an infected carcass from a choice plate. Samples with Ct >36 during amplification by qPCR were beyond the detection limit (see Methods for details) and are colored white. Mean ± SE proportion of larvae taken from carcass sites on both choice and control plates to (b) pupate and (c) eclose. Significant differences between groups are indicated by different letters. Larvae (and the subsequent pupae) were taken from male and female carcasses and varied in their infectious status, an uninfected carcass on a control plate (white bar), an uninfected carcass on a choice plate (gray bar) or an infected carcass on a choice plate (black bar). (d) The number of DCV copies present in adults derived from choice plate assays.

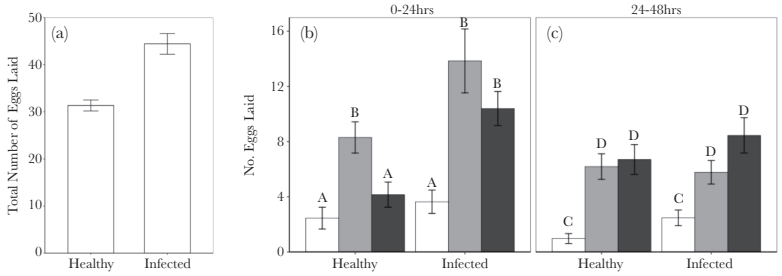


Figure 4
Adult oviposition choice. The mean ± SE number of eggs laid by infected and uninfected mothers (a) at the end of the 48-h laying period in a single oviposition arena and at the 3 oviposition sites after the (b) first 24 h of the experiment and (c) second 24-h period. Oviposition sites use the same color scheme: food only oviposition sites in white, food and uninfected carcass sites in grey and food and infected fly carcass sites in black. Significant differences between treatment groups are indicated by different letters.

foraging and adult oviposition in *D. melanogaster* are modified in the presence of potential infection by the horizontally transmitted DCV, which is known to cause a variety of physiological and behavioral pathology in fruit flies (Arnold *et al.* 2013; Chtarbanova *et al.* 2014; Stevanovic and Johnson 2015; Vale and Jardine 2015; Gupta *et al.* 2017).

Our results confirm previous findings that *Drosophila* larvae will actively cannibalize conspecific carcasses when placed in a nutrient-poor environment (Vijendravarma *et al.* 2013; Ahmad *et al.* 2015), and go further to demonstrate that necrophagy is a viable route for transmission of DCV. The consumption of infectious conspecifics, either through cannibalism or necrophagy, has been demonstrated

Table 2

Model outputs for statistical tests performed on all experiments testing the causes and costs of infection avoidance in *D. melanogaster* adult oviposition

Response	Predictor	df	*F*	*P*
Total Eggs Laid 0-48hrs	Mother Infection	1	26.6	<0.0001***
Number of Eggs Laid	Time	1	0.0702	0.79
	Ovi. Site	2	212	<0.0001***
	Mother Infection	1	0.0315	0.86
	Time × Ovi. Site	2	29.8	<0.0001***
	Time × Mother Infection	1	0.0947	0.76
	Ovi. Site × Mother Infection	2	7.37	0.0081**
	Time × Ovi. Site × Mother Infection	2	10.5	<0.0001***
Egg-to-Adult Viability	Ovi. Site	2	5.61	0.0053**
	Mother Infection	1	0.0128	0.88
	Ovi. Site × Mother Infection	2	0.528	0.592
Clutch DCV Load	Ovi. Site	2	2.55	0.0988
	Mother Infection	1	0.628	0.436
	Ovi. Site × Mother Infection	2	1.46	0.252

The oviposition site in the choice chamber is shortened to "Ovi. Site" throughout. Significant predictors are marked with asterisks (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

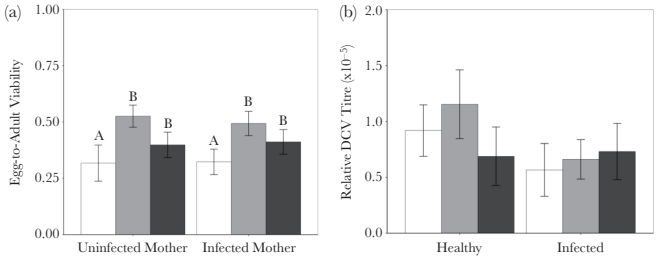


Figure 5

Adult oviposition fitness consequences. The mean ± SE (a) proportion of eggs to develop through to adulthood (egg-to-adult viability) of the clutches laid during the oviposition site choice assay and (b) the ratio of viral RNA to fly RNA ($\times 10^{-5}$) in clutches laid during the oviposition site choice assay. Oviposition sites use the same color scheme: food only oviposition sites in white, food and uninfected carcass sites in grey and food and infected fly carcass sites in black. Significant differences between treatment groups are indicated by different letters.

as a viable route of infection in a wide range of mammalian, amphibian and insect species (Forbes 2000; Qureshi *et al.* 2000; Pearman *et al.* 2004; Williams and Hernández 2006; Alpers 2008). In holometabolous insects, this phenomenon has been particularly well investigated in Lepidoptera, where cannibalism and/or necrophagy of infected conspecifics has also shown to be a viable route of transmission of several viruses during larval development (Dhandapani *et al.* 1993; Vasconcelos 1996; Boots 1998; Williams and Hernández 2006; Elvira *et al.* 2010).

Despite the risk of acquiring infection during cannibalistic foraging, we found no evidence that larval-stage flies could discriminate and avoid infectious carcasses from clean ones. Our findings contrast with a recent study in which *Drosophila* larvae showed avoidance of food contaminated with a bacterial suspension of virulent *Pseudomonas entomophila* (Surendran *et al.* 2017). In the same study, avoidance was no longer observed when using a less virulent strain of the bacterial pathogen, suggesting that external cues about the relative risk and severity of infection are key to avoidance behaviors (see also (Vale and Jardine 2017). The differences in findings likely result from different olfactory and chemo-sensory factors involved in viral and bacterial detection in *Drosophila* larvae. Furthermore, while Surendran *et al* (2017) tested evasion in 1st instar larvae, we investigated larval foraging choice during the 3rd instar, as this is the period of development when foraging activity and feeding is known to peak (Sokolowski 2001). Given that larvae are known to actively migrate towards higher quality food (Durisko and Dukas 2013), the lack of trophic infection avoidance suggests that selection for avoidance of this viral infection is weak. Weak selection for avoidance would be expected if, for example, the fitness costs of DCV infection are low during larval stage infection.

Our data is consistent with a low cost of infection in larvae, as the low titers of DCV acquired during larval feeding on infected carcasses did not have severe consequences for larval development. Our results contrast with a previous study on DCV infection of larval *D. melanogaster* which reported a 14% reduction in egg-to-adult viability, and severe mortality in adults emerged from infected larvae (Stevanovic and Johnson 2015). Unlike the relatively natural route of pathogen exposure employed in our work, larva in that study were exposed to a highly-concentrated homogenate of DCV-infected flies and exposed continuously during development until 4-days posteclosion. This difference in viral exposure may explain the more severe costs of DCV infection compared to this study.

In contrast to the lack of discrimination seen during larval foraging, we found that adult female flies do discriminate between

different types of oviposition sites. Uninfected female flies laid more eggs on sites containing an uninfected or infected carcass and food, than a site comprised only of food despite the infection risk this presents. Preference for carcass-containing sites could be explained by flies preferring to lay eggs on sites with irregular surfaces, however as uninfected mothers avoid infected carcass sites, it is more likely a result of conspecific carcasses offering additional nutrition that undermines or negates the infection risk they pose (Albeny-Simões et al. 2014). Starved *D. melanogaster* larvae assess the nutritional value of carcasses, ranging from conspecifics to natural predators (Ahmad et al. 2015), and tune their foraging strategies accordingly to optimally forage. Clutches developing on oviposition sites with a carcass present had significantly higher egg-to-adult viability than food only sites despite their significantly greater larval density (Figure 5a). The preference we see for oviposition sites containing a carcass may therefore indicate that the nutritional value of carcasses on the oviposition sites, rather than infection risk, is a greater driver of oviposition-site preference.

During the first 24 h of egg laying, uninfected flies laid significantly more eggs around uninfected carcasses. This suggests that the presence of DCV is being detected and avoided during oviposition. It is unclear which cues of DCV are detected by females, whether they are detecting the virus directly or cues of virus derived pathology in the fly carcass. Similar avoidance of pathogenic bacteria has been described in both *D. melanogaster* (Stensmyr *et al.* 2012; Babin *et al.* 2014; Kurz *et al.* 2017) and *C. elegans* (McMullan *et al.* 2012; Meisel and Kim 2014). Avoidance of virus infection has also been described in a range of invertebrates, such as gypsy moth larvae that avoid eating leaves contaminated with virus (Parker *et al.* 2010) and lobsters that avoid virus-infected conspecifics (Behringer *et al.* 2006). This avoidance likely relies on dedicated chemosensory pathways for olfactory cues (McMullan *et al.* 2012; Stensmyr *et al.* 2012; Meisel *et al.* 2014; Kurz *et al.* 2017).

In the 24–48-h period, the preference for uninfected carcasses was not observed (Figure 4c). We interpret this shift in oviposition-site preference as the result of a trade-off faced by females between minimizing DCV infection risk and maximizing fecundity. The finite nutritional value of each oviposition site dictates an optimal clutch size that each site can support. If females exceed this, fewer resources are available per offspring. As uninfected flies laid more eggs on noninfectious carcass sites in the first 24 h, the optimal clutch size is approached sooner than the other 2 sites. Fruit flies integrate the nutritional quality of oviposition sites into deciding between laying more eggs and acquiring more resources to develop more eggs (Lihoreau *et al.* 2016), a trade-off that is also seen in a range of other organisms (Blaustein 1999; Albeny-Simões et al. 2014; Tjørnløv *et al.* 2015; Lihoreau *et al.* 2016). In order to maximize the number of eggs laid, females therefore appear to risk DCV infection by laying their eggs near an infected carcass. The relative nutritional value and the potential costs of DCV infection are patent in the egg-to-adult viability of offspring from each oviposition site: the increase in viability between the food-only site and both the uninfected and infected carcass sites reflects the nutritional difference between these sites. Figure 5a suggests the benefits of oviposition near any carcass appear to outweigh the potential costs of virus infection.

In contrast to uninfected females, females infected with DCV did not discriminate between infectious and noninfectious carcasses, laying the same number of eggs in either oviposition site (Figure 4b,c). Furthermore, in the second 24-h period, infected females laid significantly more eggs at infectious carcass sites. We interpret this difference in discrimination between infected and healthy females as being driven by the mother's, rather than the offspring infection risk. For infected females already paying the cost of infection, there is little benefit to avoiding infectious sites. Further, infected females were significantly more fecund than healthy females (Figure 4a) as reported previously during infections with DCV (Gupta *et al.* 2017). Shifts to earlier or increased reproductive effort following exposure to pathogens is a widely observed host response across a range of taxa (reviewed in Duffield *et al.* 2017) including invertebrates (Creighton *et al.* 2009; Vale and Little 2012; Giehr *et al.* 2017; Gupta *et al.* 2017), birds (Blair and Webster 2007; Velando *et al.* 2006) and some mammals (Weil *et al.* 2006). An evolutionary explanation for terminal investment is that pathogen exposure is a cue of a reduction in future reproductive value and that by increasing fecundity shortly after pathogen exposure, terminal investment offsets some of the fitness costs of parasitism (Duffield *et al.* 2017).

In summary, our results show that *D. melanogaster* larvae and adults respond to infection risk differently during foraging and oviposition. Notably, oviposition site choice was affected by the female's infection status and the time-dependent nutritional value of oviposition sites. The initial DCV avoidance shown by mothers during oviposition may also explain why larvae do not avoid DCV during foraging. Alongside a relatively low cost of infection, larvae simply may not need to avoid infection because their mothers have evolved to avoid infectious sites where possible during oviposition. As larvae are not able to forage over large distances, their development—and ultimately their fitness—relies heavily on their mother's capacity to pick the environment that maximizes nutritional value while minimizing the risk of infection.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

FUNDING

This work was supported by a strategic award from the Wellcome Trust for the Centre for Immunity, Infection and Evolution (http://ciie.bio.ed.ac.uk; grant reference no. 095831). P.F.V. was supported by a Branco Weiss fellowship (https://brancoweissfellowship.org/) and a Chancellor's Fellowship (School of Biological Sciences, University of Edinburgh); J.A.S.J. was supported by a NERC E3 DTP PhD studentship.

We are grateful to H. Borthwick, H. Cowan, and A.Reid for technical assistance and media preparation. We also thank R. McMullan, C. Sarabian, and V. Curtis for constructive discussion and comments at the Royal Society meeting on "Evolution of Pathogen Avoidance Behaviours".

Conflict of Interest: The authors declare no conflict of interest.

Data accessibility: Analyses reported in this article can be reproduced using the data provided by Siva-Jothy *et al.* (2018).

Handling editor: Luke Holman

REFERENCES

Afonina I, Ankoudinova I, Mills A, Lokhov S, Huynh P, Mahoney W. 2007. Primers with 5' flaps improve real-time PCR. Biotechniques. 43:770, 772, 774.

Ahmad M, Chaudhary SU, Afzal AJ, Tariq M. 2015. Starvation-induced dietary behaviour in *Drosophila melanogaster* larvae and adults. Sci Rep. 5:14285.

Albeny-Simoes D, Murrell EG, Elliot SL, Andrade MR, Lima E, Juliano SA, Vilela EF. 2014. Attracted to the enemy: *Aedes aegypti* prefers oviposition sites with predator-killed conspecifics. Oecologia. 175:481–492.
Alpers MP. 2008. Review. The epidemiology of kuru: monitoring the epidemic from its peak to its end. Philos Trans R Soc Lond B Biol Sci. 363:3707–3713.
Arnold PA, Johnson KN, White CR. 2013. Physiological and metabolic consequences of viral infection in *Drosophila melanogaster*. J Exp Biol. 216:3350–3357.
Babin A, Kolly S, Schneider F, Dolivo V, Zini M, Kawecki TJ. 2014. Fruit flies learn to avoid odours associated with virulent infection. Biol Lett. 10:20140048.
Bates D, Maechler M, Bolker B, Walker S, Christensen RHB, Singmann H, Dai B, Scheipl F, Grothendieck G, Green P. 2018. lme4: linear mixed-effects models using 'Eigen' and S4 (accessed 15 June 2018). https://CRAN.R-project.org/package=lme4.
Behringer DC, Butler MJ, Shields JD. 2006. Ecology: avoidance of disease by social lobsters. Nature. 441:421.
Blair L, Webster JP. 2007. Dose-dependent schistosome-induced mortality and morbidity risk elevates host reproductive effort. J Evol Biol. 20:54–61.
Blaustein L. 1999. Oviposition site selection in response to risk of predation: evidence from aquatic habitats and consequences for population dynamics and community structure. In: Wasser SP, editor. Evolutionary theory and processes: modern perspectives. Netherlands: Springer. p. 441–456. http://link.springer.com/chapter/10.1007/978-94-011-4830-6_26 (accessed 29 June 2017).
Boots M. 1998. Cannibalism and the stage-dependent transmission of a viral pathogen of the Indian meal moth, *Plodia interpunctella*. Ecol Entomol. 23:118–122.
Caraguel CG, Stryhn H, Gagné N, Dohoo IR, Hammell KL. 2011. Selection of a cutoff value for real-time polymerase chain reaction results to fit a diagnostic purpose: analytical and epidemiologic approaches. J Vet. Diagn. Invest. 23:2–15.
Chtarbanova S, Lamiable O, Lee KZ, Galiana D, Troxler L, Meignin C, Hetru C, Hoffmann JA, Daeffler L, Imler JL. 2014. Drosophila C virus systemic infection leads to intestinal obstruction. J Virol. 88:14057–14069.
Creighton JC, Heflin ND, Belk MC. 2009. Cost of reproduction, resource quality, and terminal investment in a burying beetle. Am Nat. 174:673–684.
Curtis VA. 2014. Infection-avoidance behaviour in humans and other animals. Trends Immunol. 35:457–464.
Curtis V, de Barra M, Aunger R. 2011. Disgust as an adaptive system for disease avoidance behaviour. Philos Trans R Soc Lond B Biol Sci. 366:389–401.
Dhandapani N, Jayaraj S, Rabindra RJ. 1993. Cannibalism on nuclear polyhedrosis virus infected larvae by *Heliothis armigera* (Hubn.) and its effect on viral infection. Int J Trop Insect Sci. 14:427–430.
Duffield KR, Bowers EK, Sakaluk SK, Sadd BM. 2017. A dynamic threshold model for terminal investment. Behav Ecol Sociobiol. 71:185. doi:10.1007/s00265-017-2416-z. http://link.springer.com/10.1007/s00265-017-2416-z (accessed 13 June 2018)
Durisko Z, Dukas R. 2013. Attraction to and learning from social cues in fruitfly larvae. Proc Biol Sci. 280:20131398.
Elvira S, Williams T, Caballero P. 2010. Juvenile hormone analog technology: effects on larval cannibalism and the production of *Spodoptera exigua* (Lepidoptera: Noctuidae) nucleopolyhedrovirus. J Econ Entomol. 103:577–582.
Escobedo-Bonilla CM, Alday-Sanz V, Wille M, Sorgeloos P, Pensaert MB, Nauwynck HJ. 2008. A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus. J Fish Dis. 31:1–18.
Ezenwa VO, Archie EA, Craft ME, Hawley DM, Martin LB, Moore J, White L. 2016. Host behaviour–parasite feedback: an essential link between animal behaviour and disease ecology. Proc R Soc B. 283:20153078.
Forbes LB. 2000. The occurrence and ecology of Trichinella in marine mammals. Vet Parasitol. 93:321–334.
Giehr J, Grasse AV, Cremer S, Heinze J, Schrempf A. 2017. Ant queens increase their reproductive efforts after pathogen infection. R Soc Open Sci. 4:170547.
Gomariz-Zilber E, Thomas-Orillard M. 1993. Drosophila C virus and Drosophila hosts: a good association in various environments. J Evol Biol. 6:677–689.
Gupta V, Stewart CO, Rund SSC, Monteith K, Vale PF. 2017. Costs and benefits of sublethal Drosophila C virus infection. J Evol Biol. 30:1325–1335.
Hall SR, Sivars-Becker L, Becker C, Duffy MA, Tessier AJ, Cáceres CE. 2007. Eating yourself sick: transmission of disease as a function of foraging ecology. Ecol Lett. 10:207–218.
Hothorn T, Bretz F, Westfall P, Heiberger RM, Schuetzenmeister A, Scheibe S. 2017. multcomp: Simultaneous Inference in General Parametric Models. https://CRAN.R-project.org/package=multcomp (accessed 15 June 2018).
Huszar T, Imler J. 2008. Drosophila viruses and the study of antiviral host defense. Advances in virus research. 72:227–265.
Jousset FX, Bergoin M, Revet B. 1977. Characterization of the Drosophila C virus. J Gen Virol. 34:269–283.
Kacsoh BZ, Lynch ZR, Mortimer NT, Schlenke TA. 2013. Fruit flies medicate offspring after seeing parasites. Science. 339:947–950.
Kavaliers M, Choleris E, Agmo A, Pfaff DW. 2004. Olfactory-mediated parasite recognition and avoidance: linking genes to behavior. Horm Behav. 46:272–283.
Kiesecker JM, Skelly DK, Beard KH, Preisser E. 1999. Behavioral reduction of infection risk. Proc Natl Acad Sci USA. 96:9165–9168.
Klemme I, Karvonen A. 2016. Learned parasite avoidance is driven by host personality and resistance to infection in a fish–trematode interaction. Proc R Soc Lond B Biol Sci. 283:1148–1154.
Kurz CL, Charroux B, Chaduli D, Viallat-Lieutaud A, Royet J. 2017. Peptidoglycan sensing by octopaminergic neurons modulates *Drosophila* oviposition. eLife. 6: p.e21937. doi:10.7554/eLife.21937.
Lefèvre T, Chiang A, Kelavkar M, Li H, Li J, de Castillejo CL, Oliver L, Potini Y, Hunter MD, de Roode JC. 2012. Behavioural resistance against a protozoan parasite in the monarch butterfly. J Anim Ecol. 81:70–79.
Lefèvre T, de Roode JC, Kacsoh BZ, Schlenke TA. 2012. Defence strategies against a parasitoid wasp in Drosophila: fight or flight? Biol Lett. 8:230–233.
Lewis E. 2014. A new standard food medium. 1960 Drosophila Information Service. Cold Spring Harbor Protocols. 2014(9): pdb.rec081414. doi:10.1101/pdb.rec081414
Lihoreau M, Poissonnier LA, Isabel G, Dussutour A. 2016. Drosophila females trade off good nutrition with high-quality oviposition sites when choosing foods. J Exp Biol. 219:2514–2524.
McMullan R, Anderson A, Nurrish S. 2012. Behavioral and immune responses to infection require Gαq- RhoA signaling in *C. elegans*. PLoS Pathog. 8:e1002530.
Meisel JD, Kim DH. 2014. Behavioral avoidance of pathogenic bacteria by *Caenorhabditis elegans*. Trends Immunol. 35:465–470.
Meisel JD, Panda O, Mahanti P, Schroeder FC, Kim DH. 2014. Chemosensation of bacterial secondary metabolites modulates neuroendocrine signaling and behavior of *C. elegans*. Cell. 159:267–280.
Parker BJ, Barribeau SM, Laughton AM, de Roode JC, Gerardo NM. 2011. Non-immunological defense in an evolutionary framework. Trends Ecol Evol. 26:242–248.
Parker BJ, Elderd BD, Dwyer G. 2010. Host behaviour and exposure risk in an insect-pathogen interaction. J Anim Ecol. 79:863–870.
Pearman PB, Garner TW, Straub M, Greber UF. 2004. Response of the Italian agile frog (*Rana latastei*) to a Ranavirus, frog virus 3: a model for viral emergence in naïve populations. J Wildl Dis. 40:660–669.
Qureshi T, Labes RE, Lambeth M, Montgomery H, Griffin JF, Mackintosh CG. 2000. Transmission of *Mycobacterium bovis* from experimentally infected ferrets to non-infected ferrets (*Mustela furo*). N Z Vet J. 48:99–104.
de Roode JC, Lefèvre T. 2012. Behavioral immunity in insects. Insects. 3:789–820.
Schaller M, Park JH. 2011. The behavioral immune system (and why it matters). Curr Dir Psychol Sci. 20:99–103.
Sears BF, Rohr JR, Allen JE, Martin LB. 2011. The economy of inflammation: when is less more? Trends Parasitol. 27:382–387.
Shi M, Lin X-D, Tian J-H, Chen L-J, Chen X, Li C-X, Qin X-C, Li J, Cao J-P, Eden J-S, et al. 2016. Redefining the invertebrate RNA virosphere. Nature. 540:539–543.
Siva-Jothy, J.A., Montieth, K.M., Vale, P.F. 2018. Data from: navigating infection risk during oviposition and cannibalistic foraging in a holometabolous insect. Dryad Digital Repository. http://dx.doi.org/10.5061/dryad.fm3j82j
Sokolowski MB. 2001. Drosophila: genetics meets behaviour. Nat Rev Genet. 2:879–890.

Stensmyr MC, Dweck HK, Farhan A, Ibba I, Strutz A, Mukunda L, Linz J, Grabe V, Steck K, Lavista-Llanos S, et al. 2012. A conserved dedicated olfactory circuit for detecting harmful microbes in Drosophila. Cell. 151:1345–1357.
Stevanovic AL, Johnson KN. 2015. Infectivity of Drosophila C virus following oral delivery in Drosophila larvae. J Gen Virol. 96:1490–1496.
Surendran S, Huckesfeld S, Waschle B, Pankratz MJ. 2017. Pathogen-induced food evasion behavior in *Drosophila* larvae. J Exp Biol. 220:1774–1780.
Thomas-Orillard M. 1984. Modifications of mean ovariole number, fresh weight of adult females and developmental time in *Drosophila melanogaster* induced by Drosophila C virus. Genetics. 107:635–644.
Tjørnløv RS, Kissling WD, Barnagaud J-Y, Bøcher PK, Høye TT. 2015. Oviposition site selection of an endangered butterfly at local spatial scales. J Insect Conserv. 19:377–391.
Vale PF, Jardine MD. 2015. Sex-specific behavioural symptoms of viral gut infection and Wolbachia in *Drosophila melanogaster*. J Insect Physiol. 82:28–32.
Vale PF, Jardine MD. 2017. Infection avoidance behavior: viral exposure reduces the motivation to forage in female *Drosophila melanogaster*. Fly (Austin). 11:3–9.
Vale PF, Little TJ. 2012. Fecundity compensation and tolerance to a sterilizing pathogen in Daphnia. J Evol Biol. 25:1888–1896.
Vasconcelos SD. 1996. Alternative routes for the horizontal transmission of a nucleopolyhedrovirus. J Invertebr Pathol. 68:269–274.
Velando A, Drummond H, Torres R. 2006. Senescent birds redouble reproductive effort when ill: confirmation of the terminal investment hypothesis. Proc Biol Sci. 273:1443–1448.
Vijendravarma RK, Narasimha S, Kawecki TJ. 2013. Predatory cannibalism in *Drosophila melanogaster* larvae. Nat Commun. 4:1789.
Webster CL, Waldron FM, Robertson S, Crowson D, Ferrari G, Quintana JF, Brouqui JM, Bayne EH, Longdon B, Buck AH, et al. 2015. The discovery, distribution, and evolution of viruses associated with *Drosophila melanogaster*. PLoS Biol. 13:e1002210.
Weil ZM, Martin LB, Workman JL, Nelson RJ. 2006. Immune challenge retards seasonal reproductive regression in rodents: evidence for terminal investment. Biol Lett. 2:393–396.
Wickham H, RStudio. 2016. ggplot2: Create Elegant Data Visualisations Using the Grammar of Graphics. https://CRAN.R-project.org/package=ggplot2 (accessed 15 June 2018).
Wilfert L, Long G, Leggett HC, Schmid-Hempel P, Butlin R, Martin SJ, Boots M. 2016. Deformed wing virus is a recent global epidemic in honeybees driven by Varroa mites. Science. 351:594–597.
Williams T, Hernández O. 2006. Costs of cannibalism in the presence of an iridovirus pathogen of *Spodoptera frugiperda*. Ecol Entomol. 31:106–113.

7.2 Chapter 3

7.2.1 Supplementary Figures

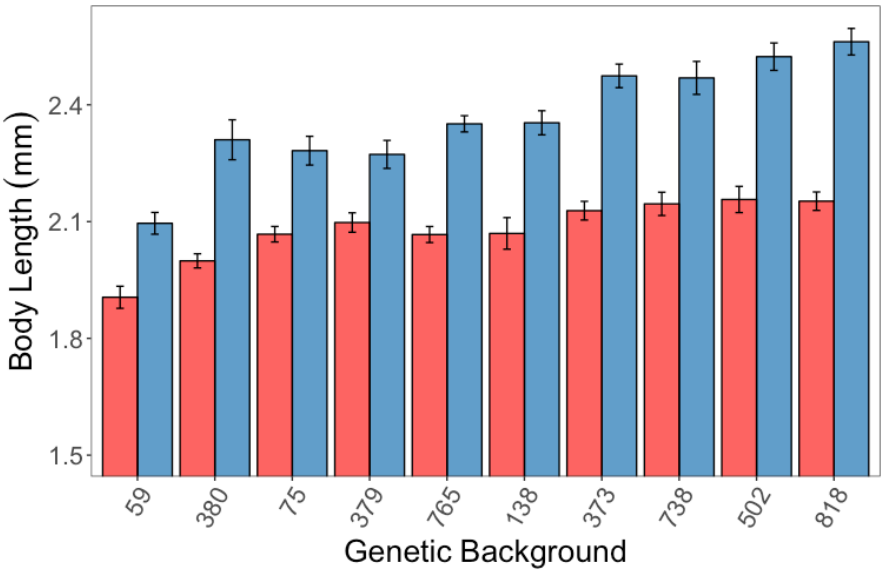


Figure S1. Mean±SE body length of male (red) and female (blue) flies calculated from 30 individuals randomly selected from each combination of sex and genetic background.

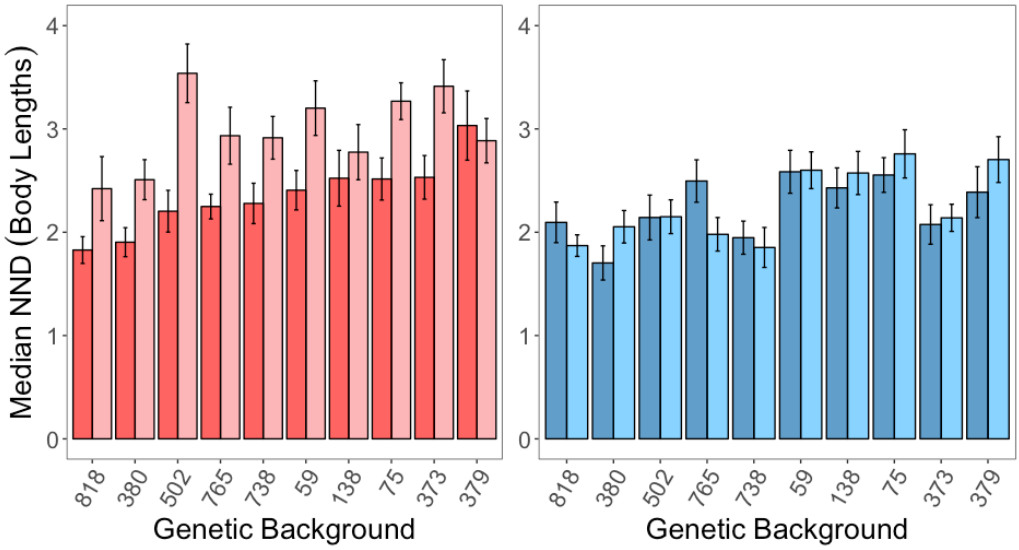


Figure S2. Mean±SE median nearest neighbour distance (NND) in body lengths of adult flies placed in Petri dishes for at least 30 minutes until settled. (a) Uninfected female-only arenas shown in blue, and infected female-only arenas in pale blue. (b) Uninfected male-only arenas are shown in red, and infected male-only arenas in pink. The x-axis of both panels is ordered from the lowest to highest mean median NND of female flies of a single genetic background.

7.2.2 Supplementary Tables

Response	Predictor	Df	F	p
Body Length	Genetic Background	9	28.5	<0.0001
	Sex	1	440.8	<0.0001
	Genetic Background * Sex	9	3.44	0.002

Table S1. Model outputs for statistical tests performed on body lengths of treatment groups comprised of each combination of sex and genetic background.

Response	Predictor	Df	F	p
Median NND (body length)	Genetic Background	9	6.55	<0.0001
	Sex	1	38.74	<0.0001
	Infection	1	24.3	<0.0001
	Genetic Background * Sex	9	1.56	0.14
	Genetic Background * Infection	9	0.99	0.54
	Sex * Infection	1	20.94	<0.0001
	Genetic Background * Sex * Infection	9	1.58	0.12

Table S2. Model outputs for statistical tests performed on social aggregation when measured using body lengths, testing the causes of variation in sociality in males and females of 10 *D. melanogaster* genetic backgrounds.

Genetic Background	Male		Female	
	Control	Infected	Control	Infected
RAL-59	24	24	22	22
RAL-75	20	27	20	20
RAL-138	21	20	18	19
RAL-373	20	26	21	28
RAL-379	20	22	21	20
RAL-380	24	20	23	24
RAL-502	23	21	21	20
RAL-738	21	21	20	20
RAL-765	26	28	20	21
RAL-818	21	20	20	22

Table S3. The sample size of each activity treatment group, representing every combination of sex, genetic background and infection status.

7.3 Chapter 4

7.3.1 Supplementary Figures

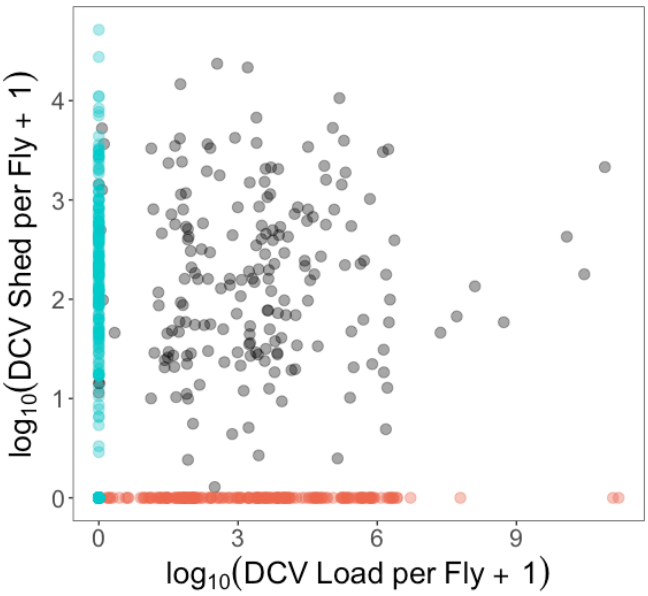


Figure S1. The relationship between the viral load of flies and the amount of virus they shed into their environment. The two distinct phenotypes, where individuals show a zero-value for shedding or load and a positive-value for the other trait, are marked by blue (supersponges) or red (supershedders).

7.3.2 Supplementary Tables

	59	75	138	373	379	380	502	738	765	818
Male	20	20	18	19	17	18	17	19	18	19
Female	17	20	13	19	19	20	19	18	15	18
Virgin Female	20	18	7	18	20	19	19	20	16	20

Table S1 – The number of flies measured for lifespan and viral load at death for each combination of genetic background and sex/female mating status.

(a)

	59	75	138	373	379	380	502	738	765	818
Male	12	11	11	12	11	11	12	11	11	6
Mated Female	13	12	11	12	12	12	11	12	12	12
Virgin Female	12	14	*NA*	15	12	13	12	12	7	12

(b)

	59	75	138	373	379	380	502	738	765	818
Male	12	12	11	12	11	12	11	12	12	6
Mated Female	14	12	11	11	12	11	11	11	12	13
Virgin Female	12	14	*NA*	14	12	11	13	10	9	12

(c)

	59	75	138	373	379	380	502	738	765	818
Male	11	12	12	12	12	11	12	10	12	7
Mated Female	11	11	10	13	12	11	13	11	12	12
Virgin Female	11	13	*NA*	13	12	13	11	12	8	11

Table S2 – The number of viral load samples for each treatment group (a) 1 DPI, (b) 2 DPI and (c) 3 DPI.

(a)

	59	75	138	373	379	380	502	738	765	818
Male	5	9	2	5	4	6	8	6	5	3
Mated Female	6	5	5	10	8	9	2	9	3	9
Virgin Female	4	11	*NA*	11	7	9	4	3	6	4

(b)

	59	75	138	373	379	380	502	738	765	818
Male	1	5	3	1	1	3	7	6	1	4
Mated Female	8	4	5	5	2	1	1	3	4	1
Virgin Female	1	3	*NA*	9	7	7	6	1	5	4

(c)

	59	75	138	373	379	380	502	738	765	818
Male	1	5	5	5	7	7	1	4	7	6
Mated Female	5	4	7	4	5	4	1	7	9	2
Virgin Female	1	1	*NA*	6	6	6	5	6	3	3

Table S3 – The number of non-zero viral load samples for each treatment group (a) 1 DPI, (b) 2 DPI and (c) 3 DPI.

(a)

	59	75	138	373	379	380	502	738	765	818
Male	10	11	10	12	11	10	12	10	10	5
Mated Female	13	12	11	11	12	12	11	12	12	12
Virgin Female	11	14	NA	15	12	13	12	12	7	12

(b)

	59	75	138	373	379	380	502	738	765	818
Male	12	12	11	11	10	12	11	12	12	7
Mated Female	14	12	11	11	12	11	11	9	12	13
Virgin Female	11	13	NA	13	12	11	13	11	9	12

(c)

	59	75	138	373	379	380	502	738	765	818
Male	10	11	12	12	11	11	12	10	9	7
Mated Female	11	9	10	13	9	9	13	8	12	11
Virgin Female	10	12	NA	13	12	12	7	11	7	10

Table S4 – The number of virus shedding samples for each treatment group (a) 1 DPI, (b) 2 DPI and (c) 3 DPI.

(a)

	59	75	138	373	379	380	502	738	765	818
Male	0	7	4	6	3	7	4	6	2	4
Mated Female	5	4	3	6	6	5	1	6	7	4
Virgin Female	4	6	NA	8	6	4	6	3	5	5

(b)

	59	75	138	373	379	380	502	738	765	818
Male	10	7	6	5	9	9	5	11	7	7
Mated Female	8	4	9	7	8	3	6	3	4	4
Virgin Female	5	6	NA	7	10	5	8	8	6	6

(c)

	59	75	138	373	379	380	502	738	765	818
Male	4	4	8	3	9	2	7	2	3	6
Mated Female	7	3	6	7	2	2	3	3	4	5
Virgin Female	4	4	NA	7	7	4	4	5	2	4

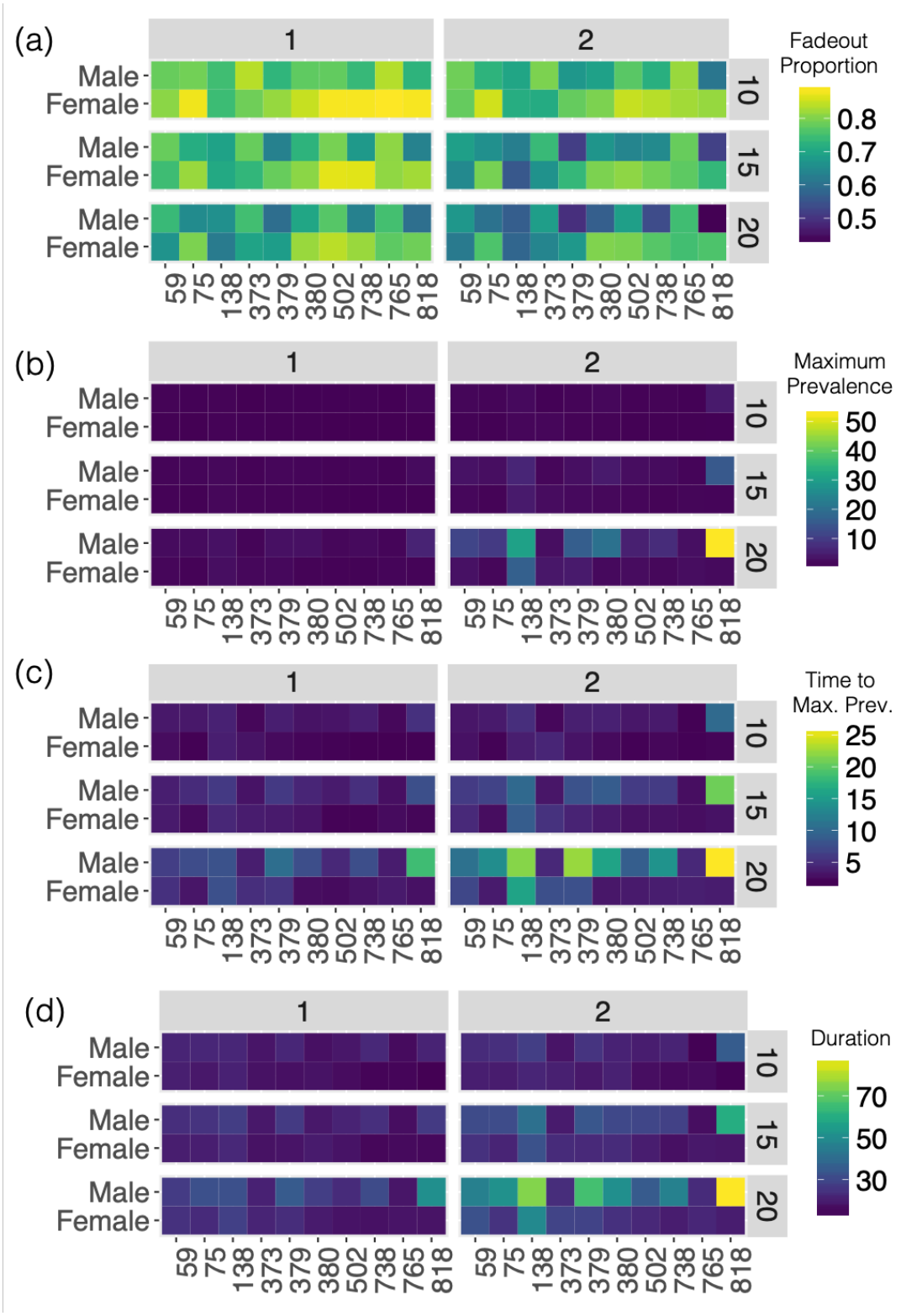
Table S5 – The number of non-zero virus shedding samples for each treatment group (a) 1 DPI, (b) 2 DPI and (c) 3 DPI.

Response Variable	Analysis	Predictors
Lifespan	GLM	Sex * Genetic Background * VLAD Mating * Genetic Background * VLAD
VLAD	GLM	Sex * Genetic Background Mating * Genetic Background
Qualitative Load	Logistic Regression	Sex * Genetic Background + DPI Mating * Genetic Background + DPI
Quantitative Load	GLM	Sex * Genetic Background + DPI Mating * Genetic Background + DPI
Qualitative Shed	Logistic Regression	Sex * Genetic Background + Quant. Load + DPI Mating * Genetic Background + Quant. Load + DPI
Quantitative Shed	GLM	Sex * Genetic Background + Quant. Load + DPI Mating * Genetic Background + Quant. Load + DPI
Qualitative *V*	Logistic Regression	Sex * Genetic Background
Quantitative *V*	GLM	Sex * Genetic Background

Table S6. Summaries of the logistic regression and GLMs used to analyse the response variables of our experiments. All interactions are fully-factorial and marked using an asterisk (*).

7.4 Chapter 5

7.4.1 Supplementary Figures



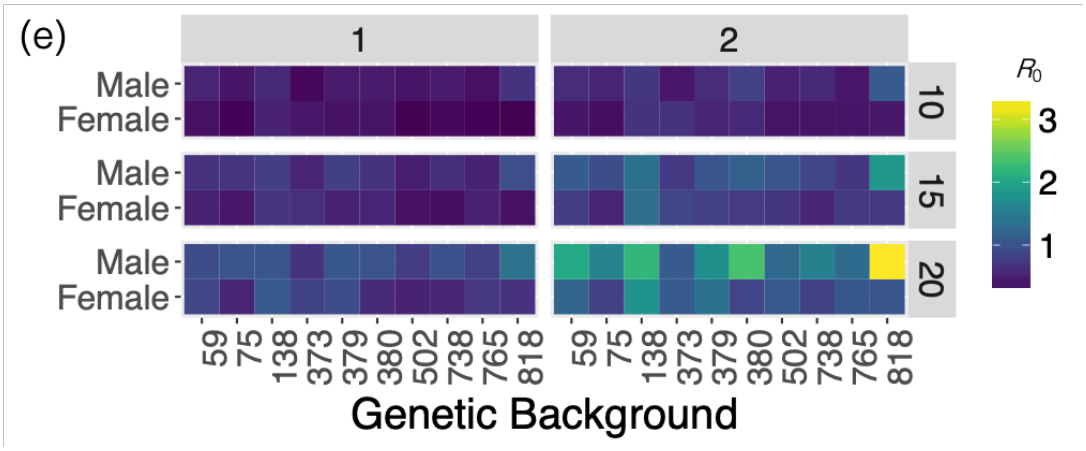
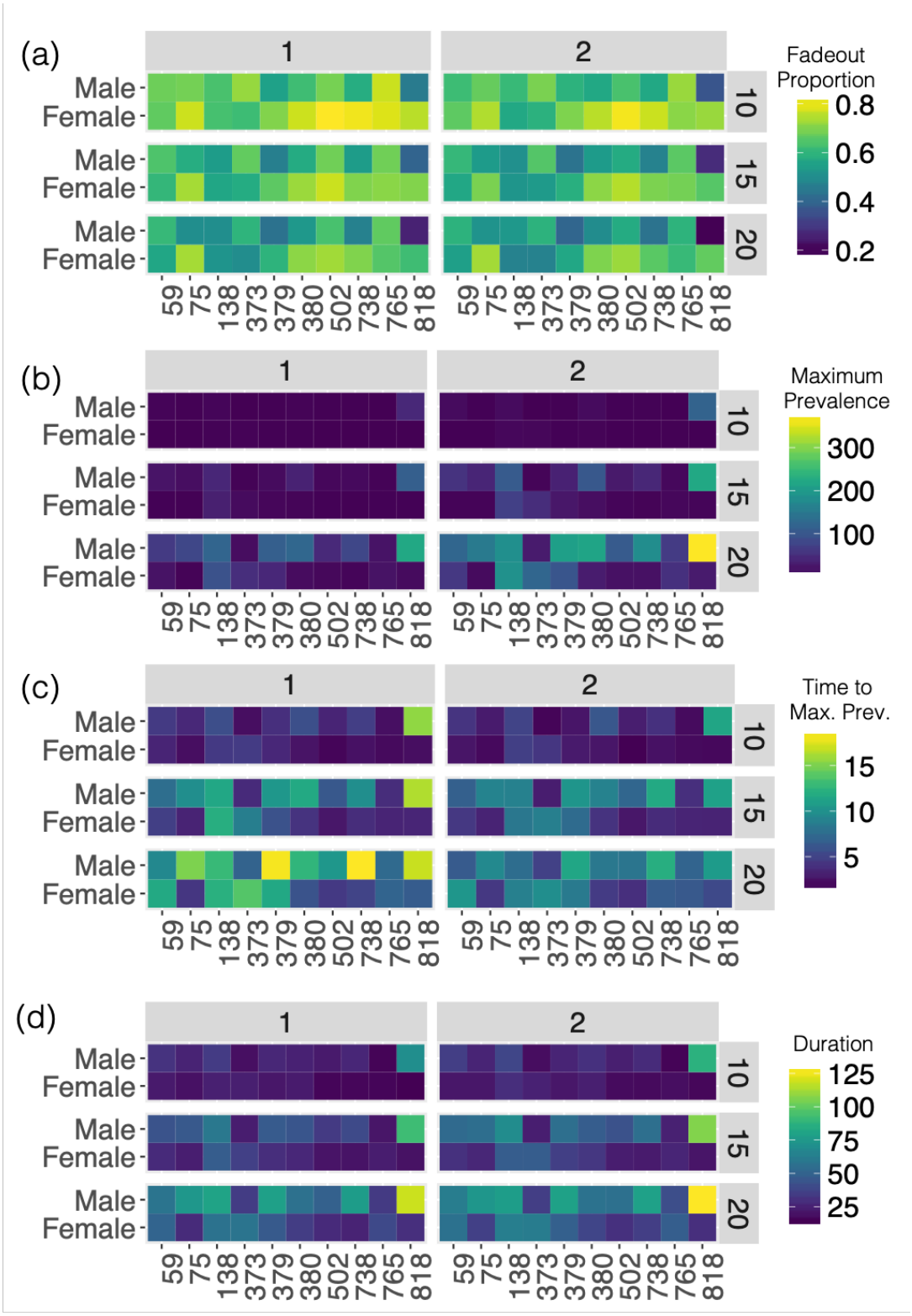


Figure S1. Theoretical experiment #1 (τ=0.1) - Mean (a) fadeout proportion, (b) maximum number of infected individuals (maximum prevalence), (c) the number of time steps taken to reach the maximum number of infected individuals (time to Max. Prev.) (d) the number of time steps outbreaks lasted for (duration) and (e) the number of secondary infections caused by the first infected individual (R_0) of outbreaks simulated in theoretical populations in experiment #1. Theoretical populations were comprised of individuals of the same sex and genetic background. Across these simulations, pathogen transmission efficiency (τ) was 0.1, infectiousness (η) was scaled by 1 or 2 (x-axis facets) and contact network connections were based on flies aggregating within 10, 15 or 20mm of one another (r; y-axis facets).


(a)
1
2
Male
Female
10
15
20
59
75
138
373
379
380
502
738
765
818
Fadeout Proportion
0.8
0.6
0.4
0.2
(b)
Maximum Prevalence
300
200
100
(c)
Time to Max. Prev.
15
10
5
(d)
Duration
125
100
75
50
25

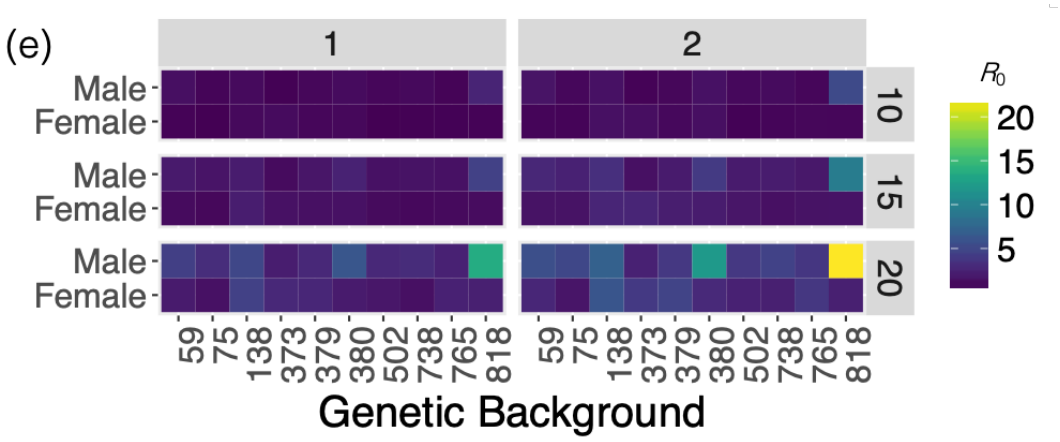
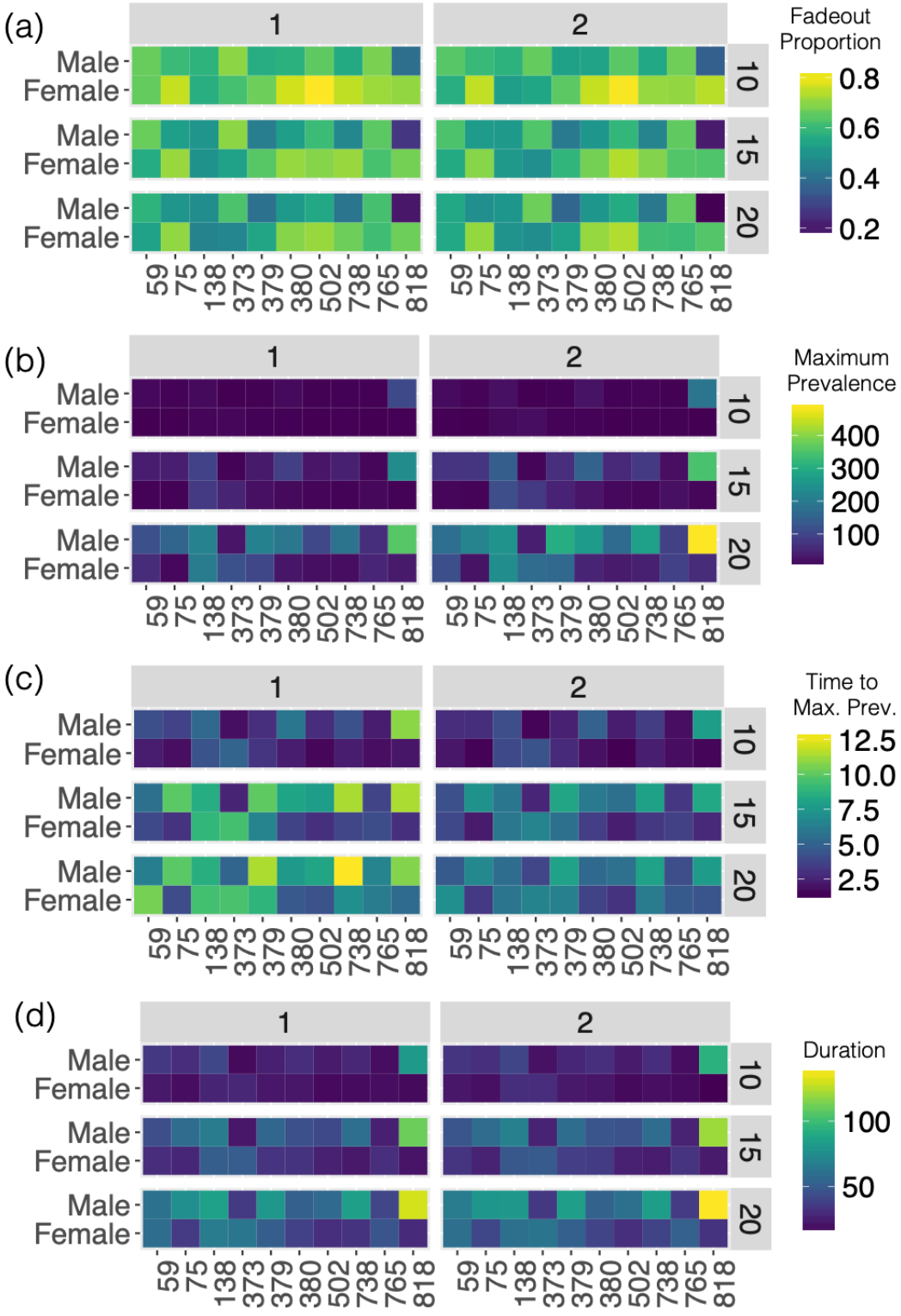


Figure S2. Theoretical experiment #1 (τ=0.5) - Mean (a) fadeout proportion, (b) maximum number of infected individuals (maximum prevalence), (c) the number of time steps taken to reach the maximum number of infected individuals (time to Max. Prev.) (d) the number of time steps outbreaks lasted for (duration) and (e) the number of secondary infections caused by the first infected individual (R_0) of outbreaks simulated in theoretical populations in experiment #1. Theoretical populations were comprised of individuals of the same sex and genetic background. Across these simulations, pathogen transmission efficiency (τ) was 0.1, infectiousness (η) was scaled by 1 or 2 (x-axis facets) and contact network connections were based on flies aggregating within 10, 15 or 20mm of one another (r; y-axis facets).


(a)
1
2
Male
Female
10
15
20
59
75
138
373
379
380
502
738
765
818
Fadeout Proportion
0.8
0.6
0.4
0.2
(b)
Maximum Prevalence
400
300
200
100
(c)
Time to Max. Prev.
12.5
10.0
7.5
5.0
2.5
(d)
Duration
100
50

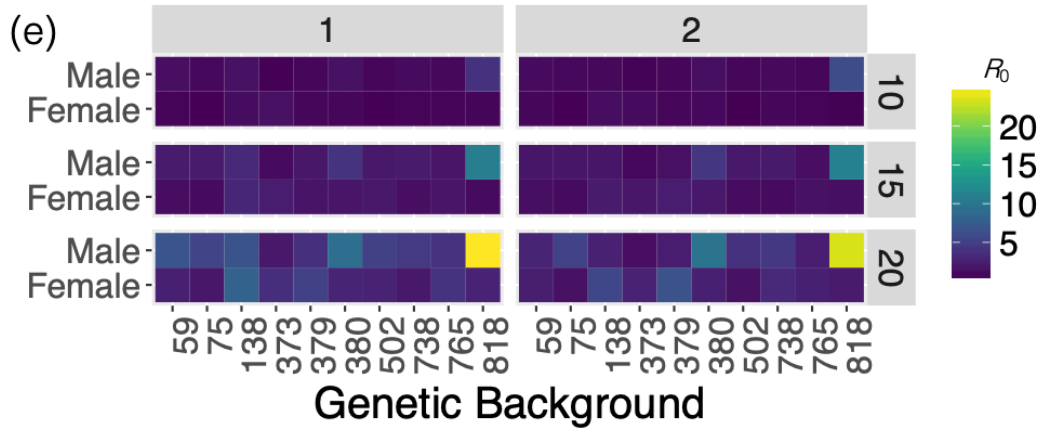
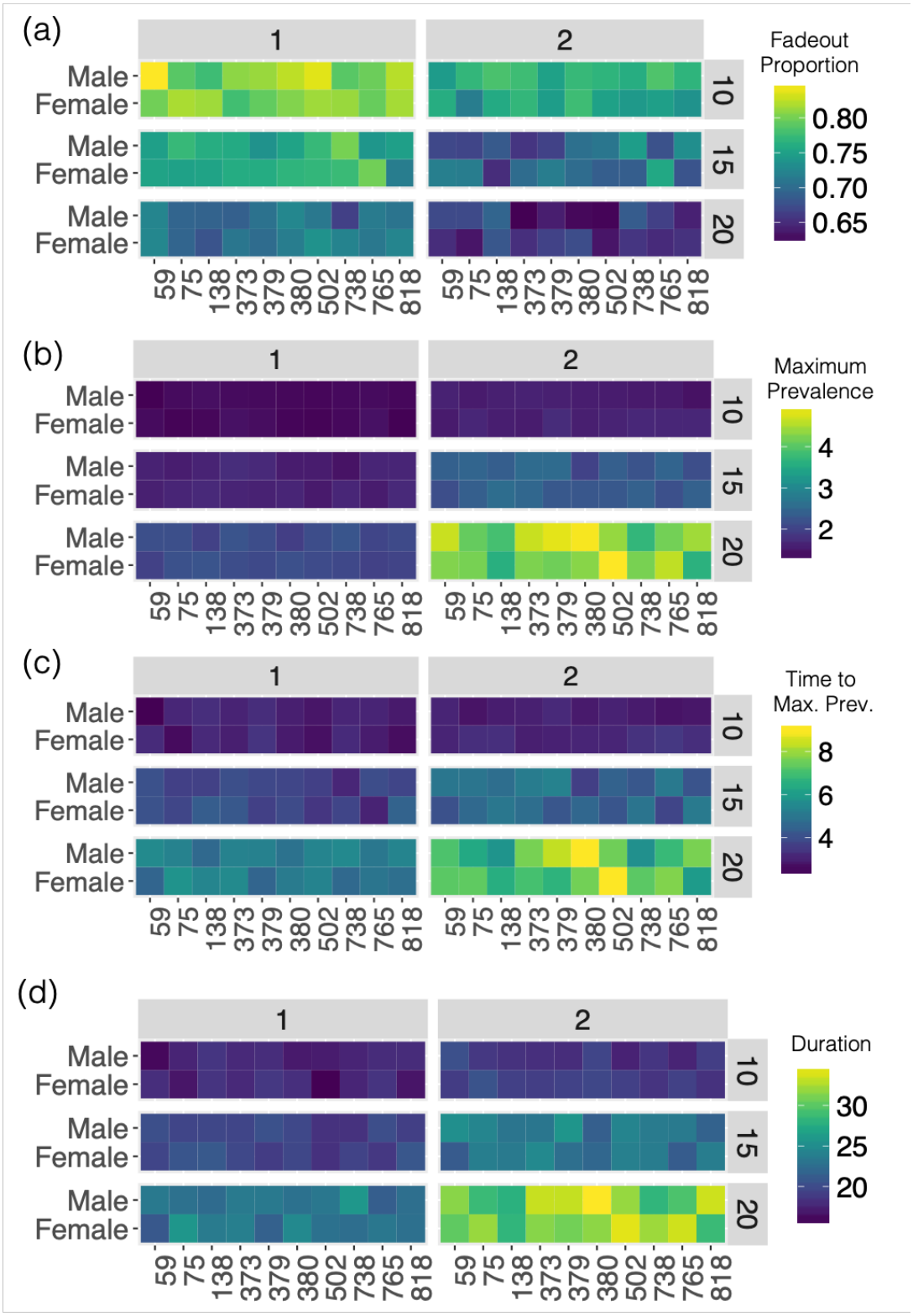


Figure S3. Theoretical experiment #1 (τ=1.0) - Mean (a) fadeout proportion, (b) maximum number of infected individuals (maximum prevalence), (c) the number of time steps taken to reach the maximum number of infected individuals (time to Max. Prev.) (d) the number of time steps outbreaks lasted for (duration) and (e) the number of secondary infections caused by the first infected individual (R_0) of outbreaks simulated in theoretical populations in experiment #1. Theoretical populations were comprised of individuals of the same sex and genetic background. Across these simulations, pathogen transmission efficiency (τ) was 0.1, infectiousness (η) was scaled by 1 or 2 (x-axis facets) and contact network connections were based on flies aggregating within 10, 15 or 20mm of one another (r; y-axis facets).


(a)
1
2
Male
Female
10
15
20
59
75
138
373
379
380
502
738
765
818
Fadeout Proportion
0.80
0.75
0.70
0.65
(b)
Maximum Prevalence
4
3
2
(c)
Time to Max. Prev.
8
6
4
(d)
Duration
30
25
20

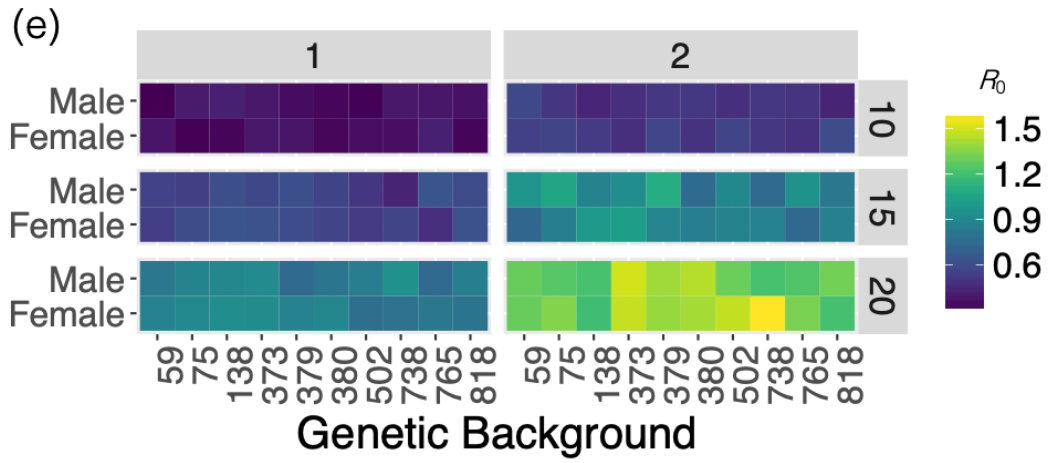
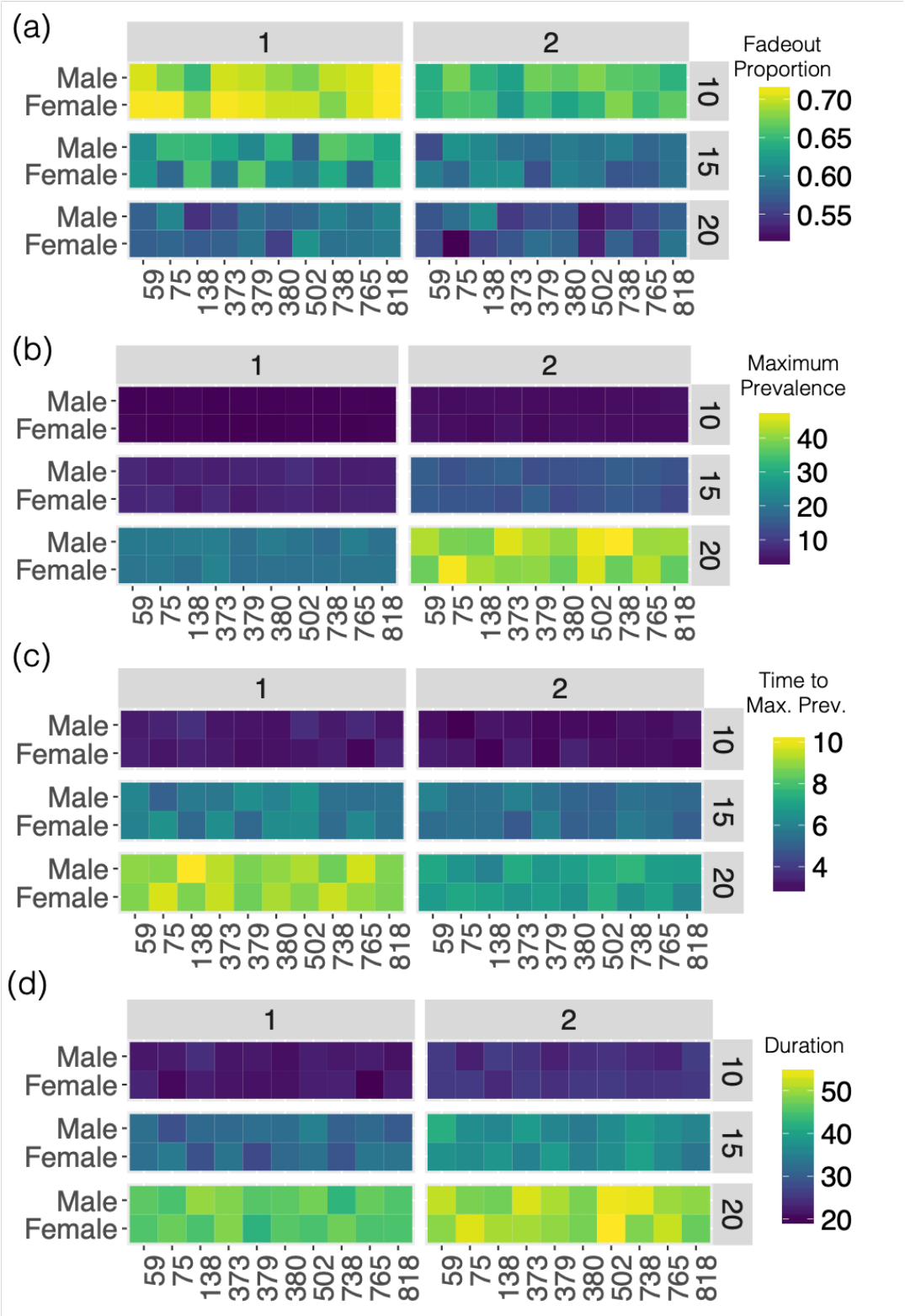


Figure S4. Theoretical experiment #2 (τ=0.1) - Mean (a) fadeout proportion, (b) maximum number of infected individuals (maximum prevalence), (c) the number of time steps taken to reach the maximum number of infected individuals (time to Max. Prev.) (d) the number of time steps outbreaks lasted for (duration) and (e) the number of secondary infections caused by the first infected individual (R_0) of outbreaks simulated in theoretical populations in experiment #2. Theoretical populations were comprised of 50 individuals from each combination of sex and genetic background. Across these simulations, pathogen transmission efficiency (τ) was 0.1, infectiousness (η) was scaled by 1 or 2 (x-axis facets) and contact network connections were based on flies aggregating within 10, 15 or 20mm of one another (r; y-axis facets).


(a)
1
2
Male
Female
10
15
20
59
75
138
373
379
380
502
738
765
818
Fadeout Proportion
0.70
0.65
0.60
0.55
(b)
Maximum Prevalence
40
30
20
10
(c)
Time to Max. Prev.
10
8
6
4
(d)
Duration
50
40
30
20

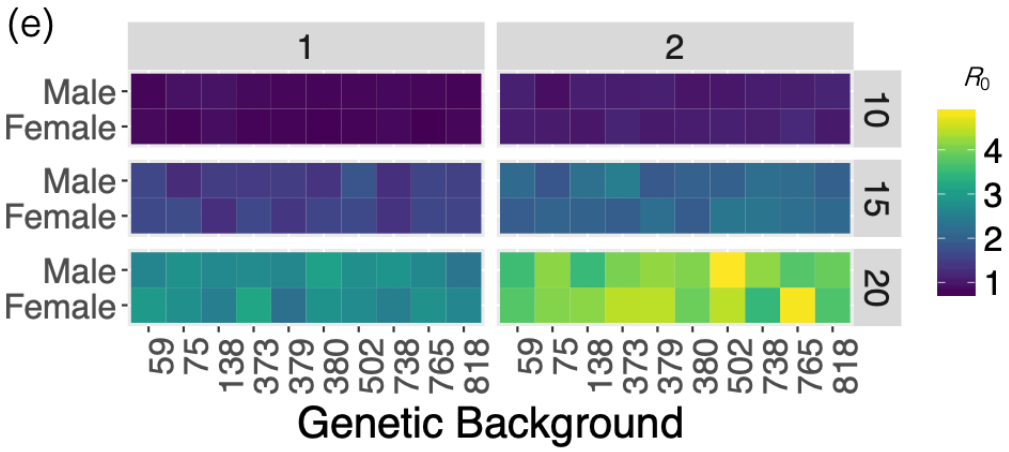
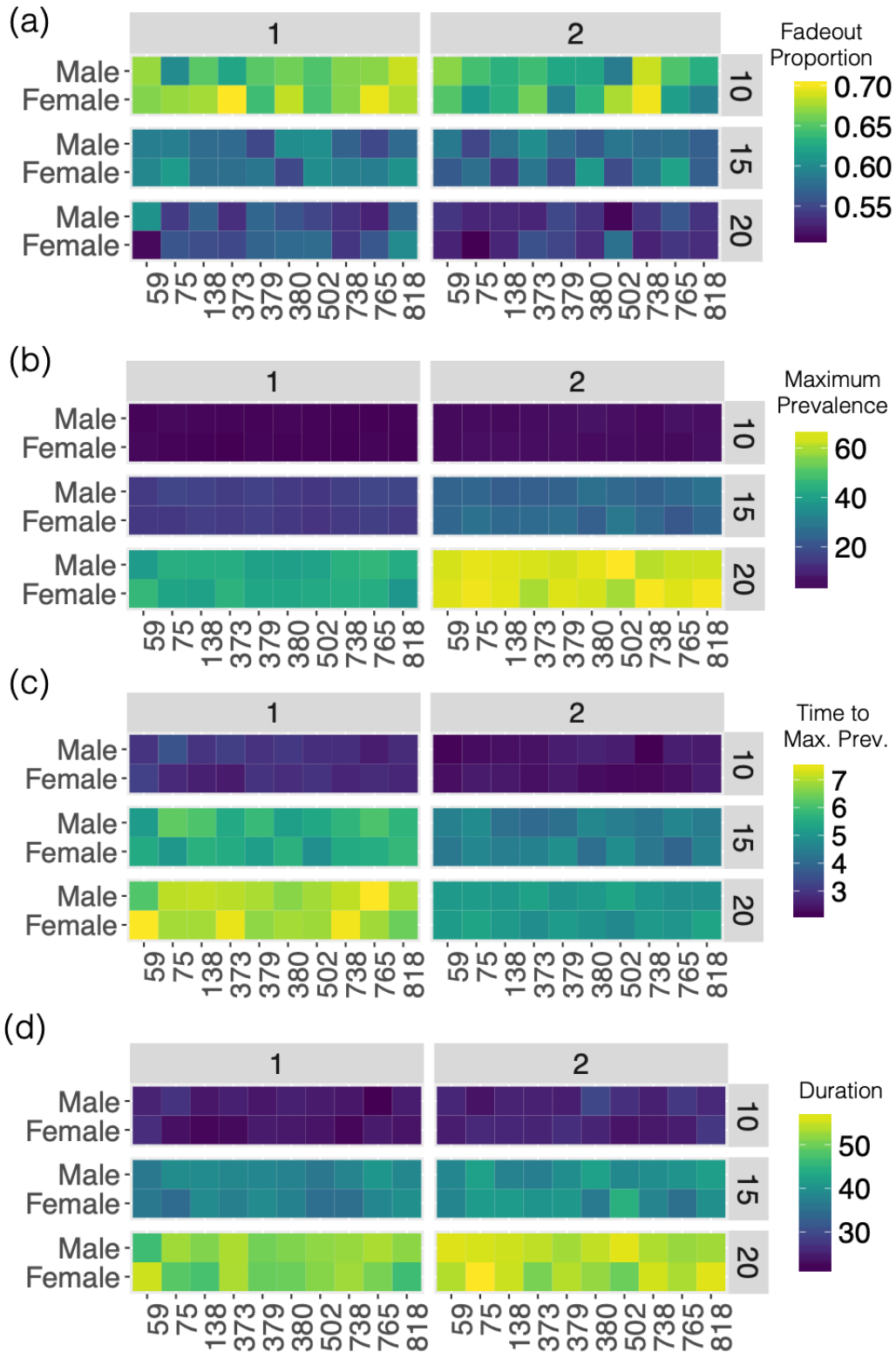


Figure S5. Theoretical experiment #2 (τ=0.5) - Mean (a) fadeout proportion, (b) maximum number of infected individuals (maximum prevalence), (c) the number of time steps taken to reach the maximum number of infected individuals (time to Max. Prev.) (d) the number of time steps outbreaks lasted for (duration) and (e) the number of secondary infections caused by the first infected individual (R_0) of outbreaks simulated in theoretical populations in experiment #2. Theoretical populations were comprised of 50 individuals from each combination of sex and genetic background. Across these simulations, pathogen transmission efficiency (τ) was 0.5, infectiousness (η) was scaled by 1 or 2 (x-axis facets) and contact network connections were based on flies aggregating within 10, 15 or 20mm of one another (r; y-axis facets).


(a)
1
2
Male
Female
10
15
20
59
75
138
373
379
380
502
738
765
818
Fadeout Proportion
0.70
0.65
0.60
0.55
(b)
Maximum Prevalence
60
40
20
(c)
Time to Max. Prev.
7
6
5
4
3
(d)
Duration
50
40
30

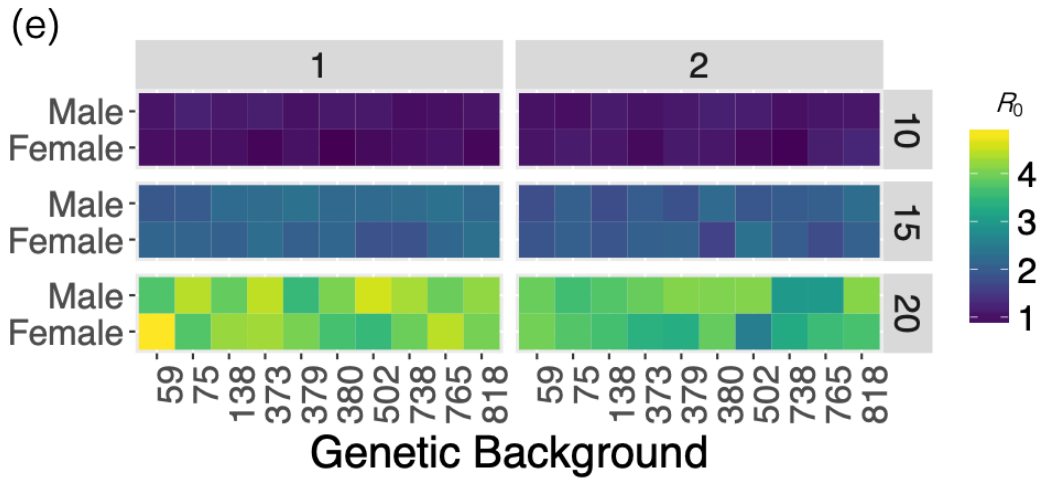


Figure S6. Theoretical experiment #2 (τ=1.0) - Mean (a) fadeout proportion, (b) maximum number of infected individuals (maximum prevalence), (c) the number of time steps taken to reach the maximum number of infected individuals (time to Max. Prev.) (d) the number of time steps outbreaks lasted for (duration) and (e) the number of secondary infections caused by the first infected individual (R_0) of outbreaks simulated in theoretical populations in experiment #2. Theoretical populations were comprised of 50 individuals from each combination of sex and genetic background. Across these simulations, pathogen transmission efficiency (τ) was 1.0, infectiousness (η) was scaled by 1 or 2 (x-axis facets) and contact network connections were based on flies aggregating within 10, 15 or 20mm of one another (r; y-axis facets).

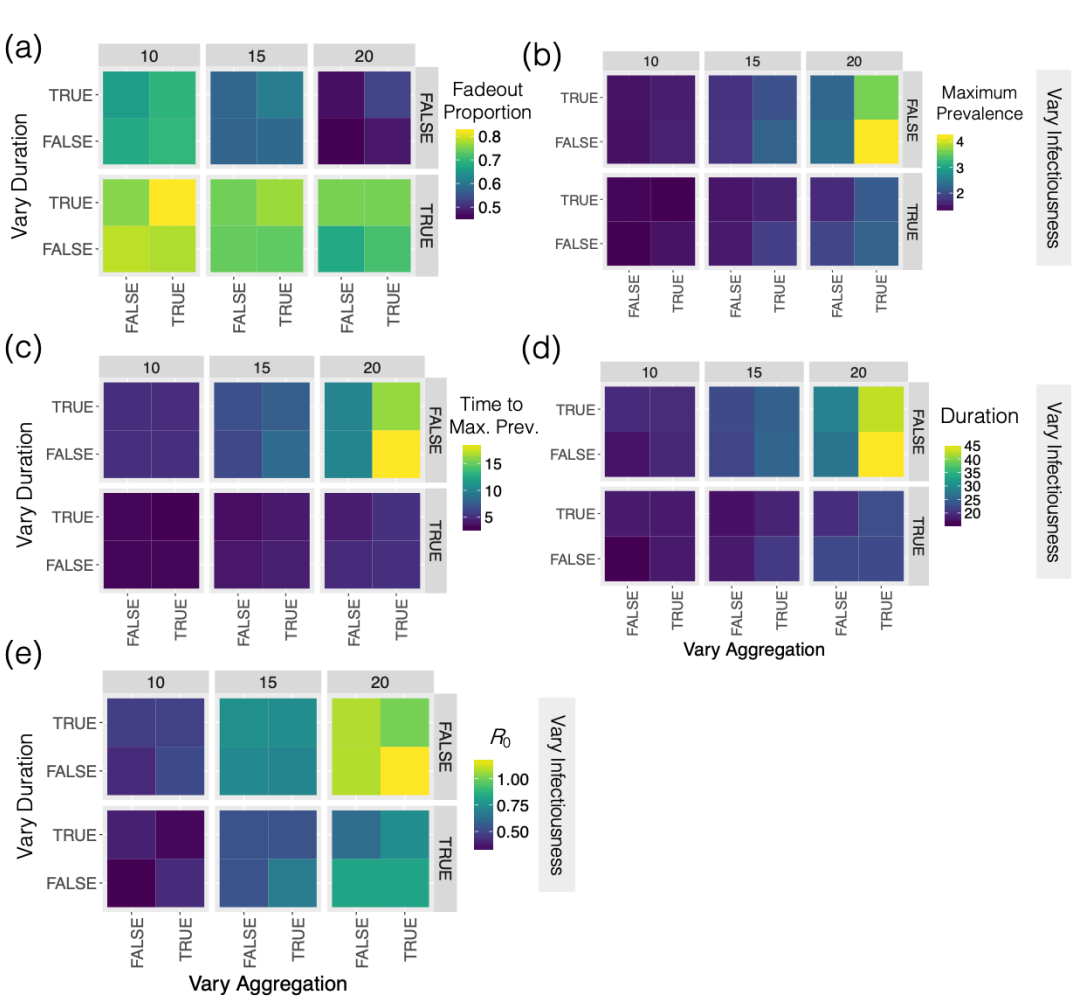


Figure S7. Theoretical experiment #3 (τ=0.1, η=1) - Mean (a) fadeout proportion, (b) maximum number of infected individuals (maximum prevalence), (c) the number of time steps taken to reach the maximum number of infected individuals (time to Max. Prev.) (d) the number of time steps outbreaks lasted for (duration) and (e) the number of secondary infections caused by the first infected individual (R_0) of outbreaks simulated in theoretical populations in experiment #3. Theoretical populations were comprised of individuals whose virus shedding, social aggregation and lifespan were derived from the variation seen across all genetic backgrounds and both sexes. The extent of this variation was manipulated by constraining variation in social aggregation (vary aggregation; x-axis), lifespan following infection (vary duration; y-axis) and virus shedding (vary infectiousness; y-axis facets) to the population mean. Across these simulations, pathogen transmission efficiency (τ) was 0.1, infectiousness (η) was 1, and contact network connections were based on flies aggregating within 10, 15 or 20mm of one another (r; x-axis facets).

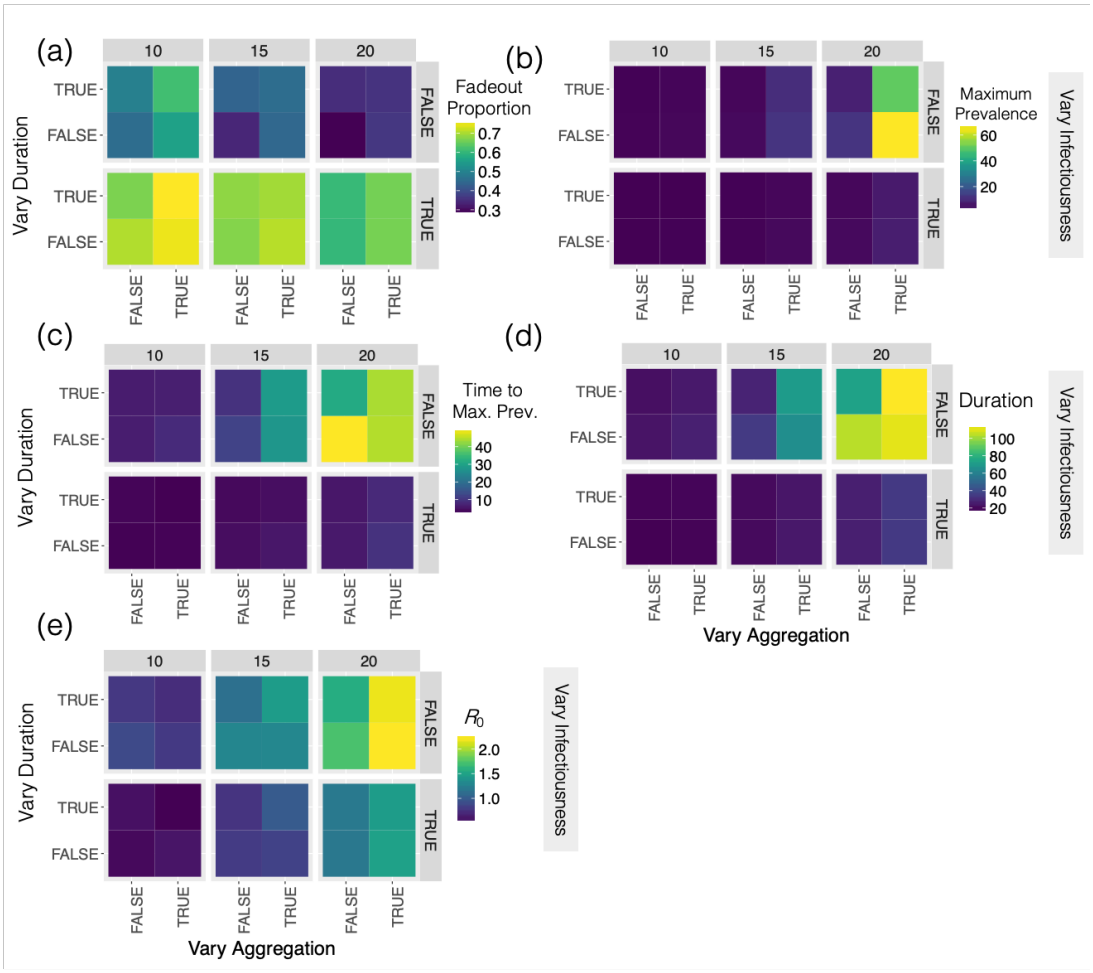


Figure S8. Theoretical experiment #3 (τ=0.1, η=2) - Mean (a) fadeout proportion, (b) maximum number of infected individuals (maximum prevalence), (c) the number of time steps taken to reach the maximum number of infected individuals (time to Max. Prev.) (d) the number of time steps outbreaks lasted for (duration) and (e) the number of secondary infections caused by the first infected individual (R_0) of outbreaks simulated in theoretical populations in experiment #3. Theoretical populations were comprised of individuals whose virus shedding, social aggregation and lifespan were derived from the variation seen across all genetic backgrounds and both sexes. The extent of this variation was manipulated by constraining variation in social aggregation (vary aggregation; x-axis), lifespan following infection (vary duration; y-axis) and virus shedding (vary infectiousness; y-axis facets) to the population mean. Across these simulations, pathogen transmission efficiency (τ) was 0.1, infectiousness (η) was 2, and contact network connections were based on flies aggregating within 10, 15 or 20mm of one another (r; x-axis facets).

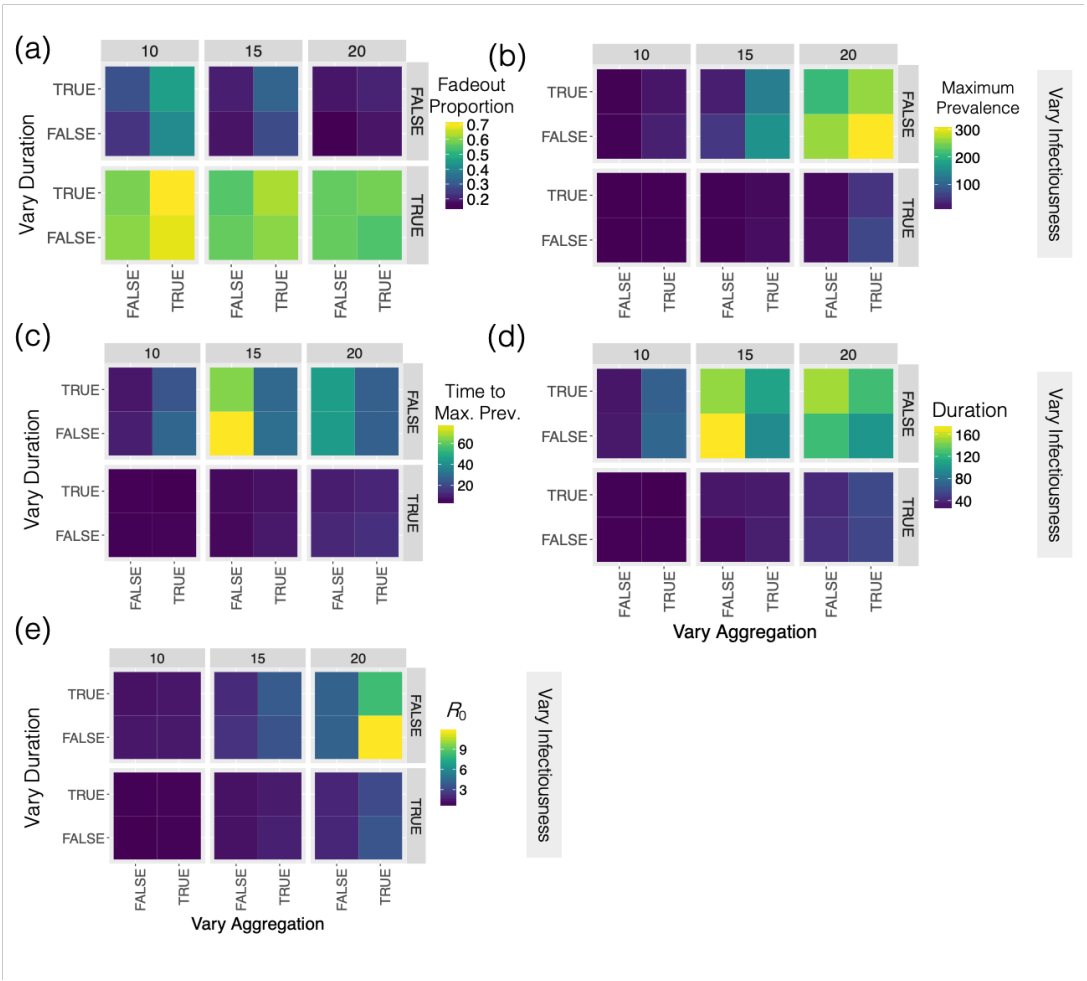


Figure S9. Theoretical experiment #3 (τ=0.5, η=1) - Mean (a) fadeout proportion, (b) maximum number of infected individuals (maximum prevalence), (c) the number of time steps taken to reach the maximum number of infected individuals (time to Max. Prev.) (d) the number of time steps outbreaks lasted for (duration) and (e) the number of secondary infections caused by the first infected individual (R_0) of outbreaks simulated in theoretical populations in experiment #3. Theoretical populations were comprised of individuals whose virus shedding, social aggregation and lifespan were derived from the variation seen across all genetic backgrounds and both sexes. The extent of this variation was manipulated by constraining variation in social aggregation (vary aggregation; x-axis), lifespan following infection (vary duration; y-axis) and virus shedding (vary infectiousness; y-axis facets) to the population mean. Across these simulations, pathogen transmission efficiency (τ) was 0.5, infectiousness (η) was 1 and contact network connections were based on flies aggregating within 10, 15 or 20mm of one another (r; x-axis facets).

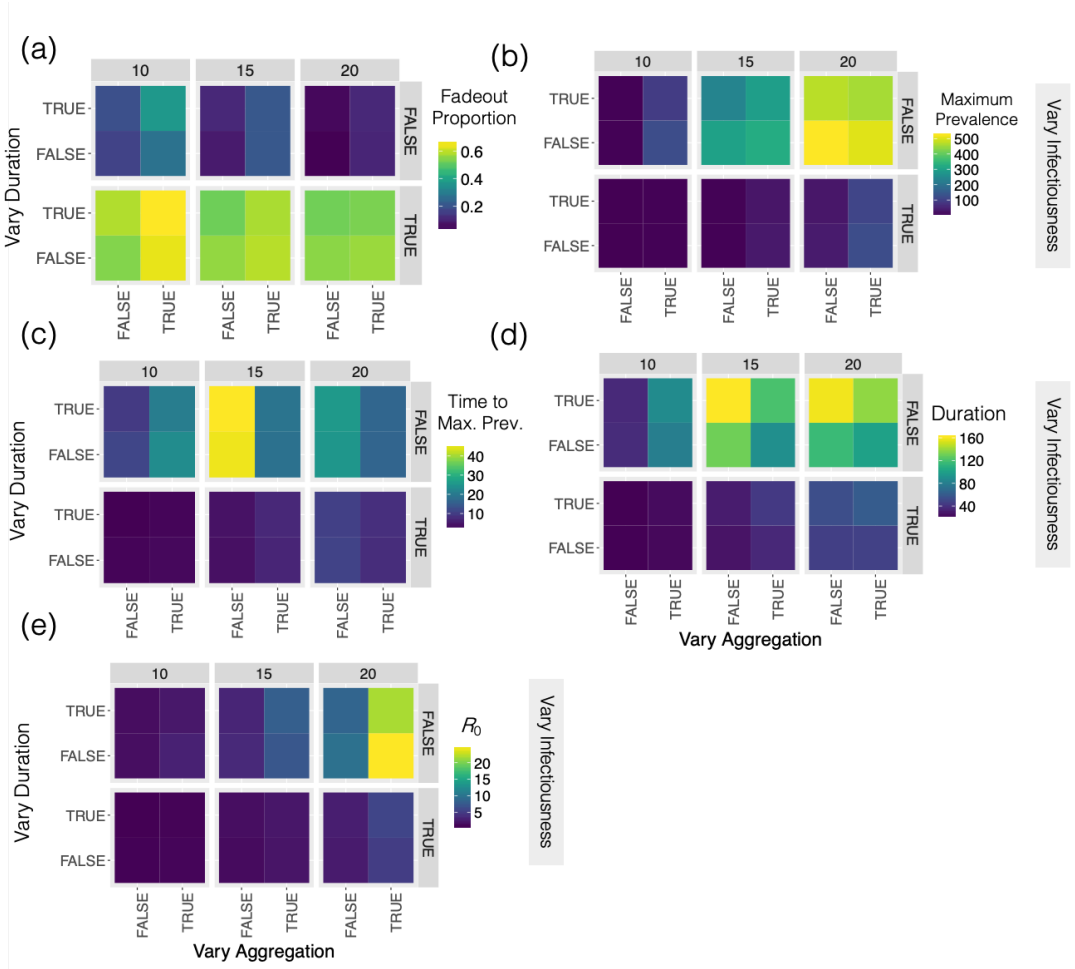


Figure S10. Theoretical experiment #3 (τ=0.5, η=2) - Mean (a) fadeout proportion, (b) maximum number of infected individuals (maximum prevalence), (c) the number of time steps taken to reach the maximum number of infected individuals (time to Max. Prev.) (d) the number of time steps outbreaks lasted for (duration) and (e) the number of secondary infections caused by the first infected individual (R_0) of outbreaks simulated in theoretical populations in experiment #3. Theoretical populations were comprised of individuals whose virus shedding, social aggregation and lifespan were derived from the variation seen across all genetic backgrounds and both sexes. The extent of this variation was manipulated by constraining variation in social aggregation (vary aggregation; x-axis), lifespan following infection (vary duration; y-axis) and virus shedding (vary infectiousness; y-axis facets) to the population mean. Across these simulations, pathogen transmission efficiency (τ) was 0.5, infectiousness (η) was 2 and contact network connections were based on flies aggregating within 10, 15 or 20mm of one another (r; x-axis facets).

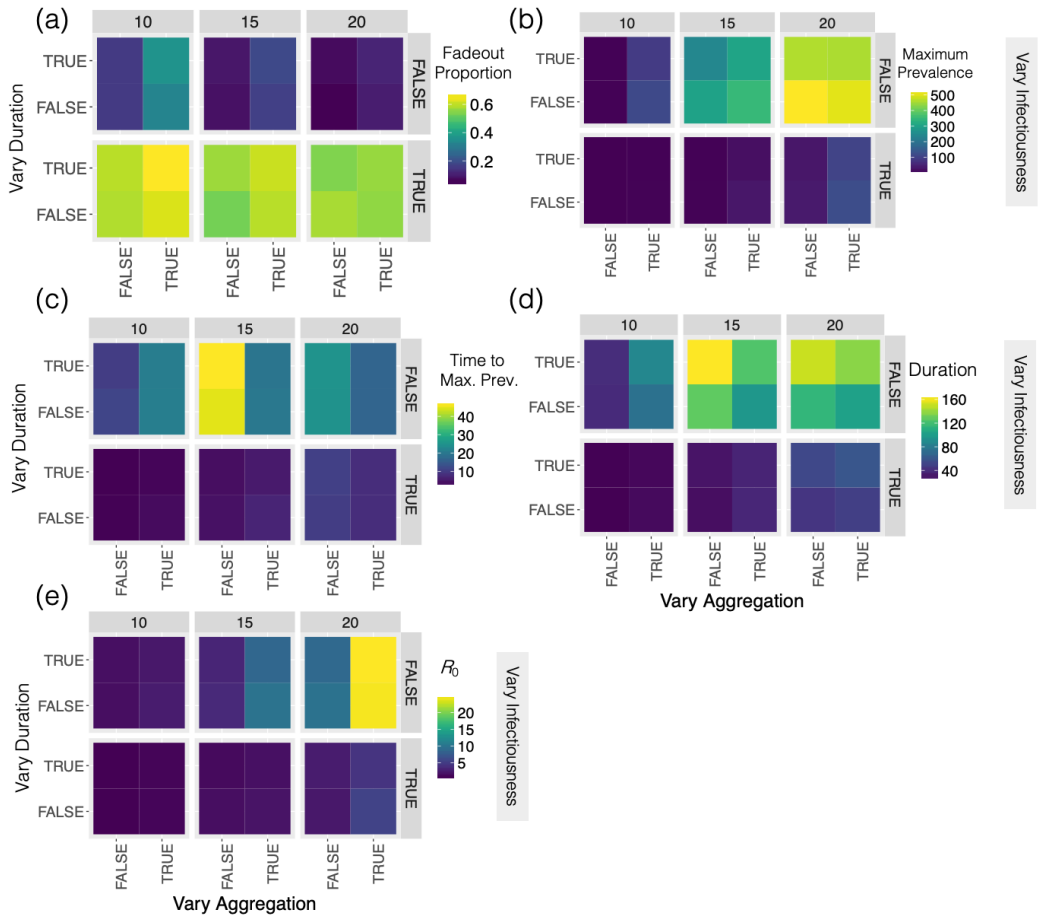


Figure S11. Theoretical experiment #3 (τ=1.0, η=1) - Mean (a) fadeout proportion, (b) maximum number of infected individuals (maximum prevalence), (c) the number of time steps taken to reach the maximum number of infected individuals (time to Max. Prev.) (d) the number of time steps outbreaks lasted for (duration) and (e) the number of secondary infections caused by the first infected individual (R_0) of outbreaks simulated in theoretical populations in experiment #3. Theoretical populations were comprised of individuals whose virus shedding, social aggregation and lifespan were derived from the variation seen across all genetic backgrounds and both sexes. The extent of this variation was manipulated by constraining variation in social aggregation (vary aggregation; x-axis), lifespan following infection (vary duration; y-axis) and virus shedding (vary infectiousness; y-axis facets) to the population mean. Across these simulations, pathogen transmission efficiency (τ) was 1.0, infectiousness (η) was 1 and contact network connections were based on flies aggregating within 10, 15 or 20mm of one another (r; x-axis facets).

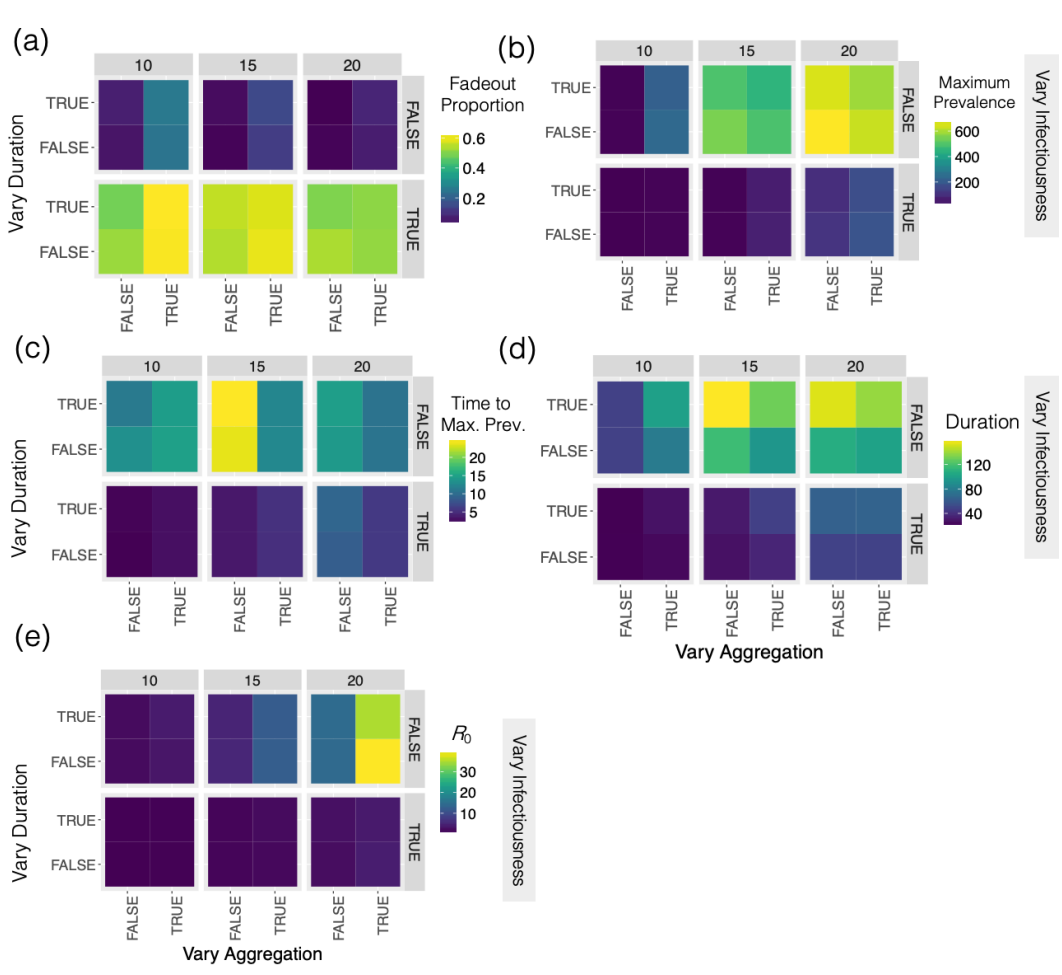


Figure S12. Theoretical experiment #3 (τ=1.0, η=2) - Mean (a) fadeout proportion, (b) maximum number of infected individuals (maximum prevalence), (c) the number of time steps taken to reach the maximum number of infected individuals (time to Max. Prev.) (d) the number of time steps outbreaks lasted for (duration) and (e) the number of secondary infections caused by the first infected individual (R_0) of outbreaks simulated in theoretical populations in experiment #3. Theoretical populations were comprised of individuals whose virus shedding, social aggregation and lifespan were derived from the variation seen across all genetic backgrounds and both sexes. The extent of this variation was manipulated by constraining variation in social aggregation (vary aggregation; x-axis), lifespan following infection (vary duration; y-axis) and virus shedding (vary infectiousness; y-axis facets) to the population mean. Across these simulations, pathogen transmission efficiency (τ) was 1.0, infectiousness (η) was 2 and contact network connections were based on flies aggregating within 10, 15 or 20mm of one another (r; x-axis facets).

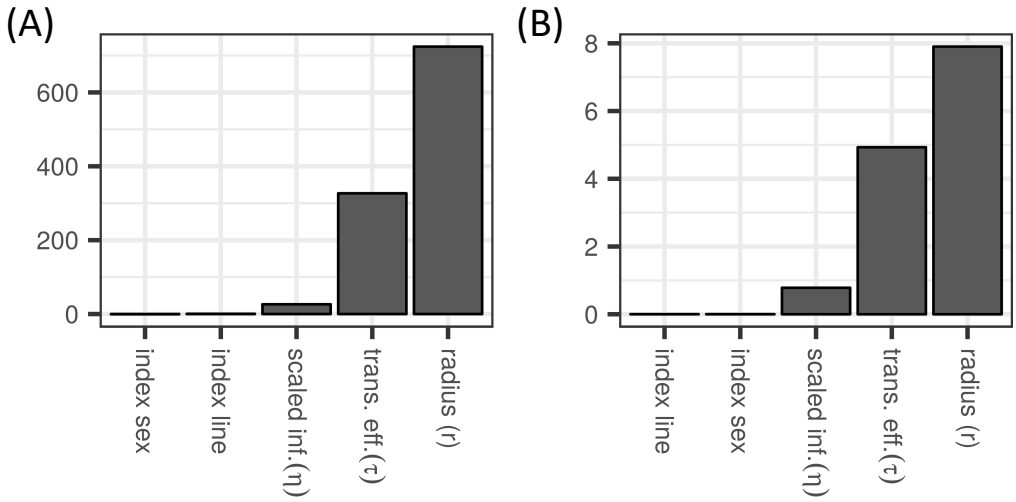


Figure S13. Theoretical Experiment #2, (r=15, τ=1.0, η=2). Results of variable importance analysis for Experiment #2 with n=1000 trees using *cforest* function from the *party* package in R. Variables are listed on x-axis. Y-axis describes variable importance (mean decrease in accuracy [MDA]). Which variables most determine (A) how long an infection lasts, and (B) the number of secondary cases of infection caused by the first individual to become infected?

7.5 Other Publications

I contributed to the following works during my PhD, however they are not part of my thesis.

7.5.1 The route of infection determines *Wolbachia* antibacterial protection in *Drosophila*

Gupta V, Vasanthakrishnan RB, Siva-Jothy J, Monteith KM, Brown SP, Vale PF. (2017) The route of infection determines *Wolbachia* antibacterial protection in *Drosophila*. *Proceedings of the Royal Society B: Biological Sciences*. 284: 20170809.

PROCEEDINGS B

rspb.royalsocietypublishing.org

Research

Cite this article: Gupta V, Vasanthakrishnan RB, Siva-Jothy J, Monteith KM, Brown SP, Vale PF. 2017 The route of infection determines *Wolbachia* antibacterial protection in *Drosophila*. *Proc. R. Soc. B* **284**: 20170809. http://dx.doi.org/10.1098/rspb.2017.0809

Received: 14 April 2017
Accepted: 12 May 2017

Subject Category:
Development and physiology

Subject Areas:
microbiology, immunology, ecology

Keywords:
Wolbachia, invertebrate immunity, *Drosophila*, symbiont protection, infection tolerance, sexual dimorphism

Author for correspondence:
Pedro F. Vale
e-mail: pedro.vale@ed.ac.uk

†These authors contributed equally to this study.

Electronic supplementary material is available online at https://dx.doi.org/10.6084/m9.figshare.c.3787412.

THE ROYAL SOCIETY PUBLISHING

The route of infection determines *Wolbachia* antibacterial protection in *Drosophila*

Vanika Gupta[1,†], Radhakrishnan B. Vasanthakrishnan[3,†], Jonathon Siva-Jothy[1], Katy M. Monteith[1], Sam P. Brown[4] and Pedro F. Vale[1,2]

[1]Institute of Evolutionary Biology, School of Biological Sciences, and [2]Centre for Immunity, Infection and Evolution, University of Edinburgh, Edinburgh EH9 3FL, UK
[3]IGDR-CNRS UMR 6290, 2, Avenue Du Professeur Léon Bernard, 35043 Rennes, France
[4]School of Biology, Georgia Institute of Technology, Atlanta, GA 30332-0230, USA

PFV, 0000-0003-4558-9202

Bacterial symbionts are widespread among metazoans and provide a range of beneficial functions. *Wolbachia*-mediated protection against viral infection has been extensively demonstrated in *Drosophila*. In mosquitoes that are artificially transinfected with *Drosophila melanogaster Wolbachia* (wMel), protection from both viral and bacterial infections has been demonstrated. However, no evidence for *Wolbachia*-mediated antibacterial protection has been demonstrated in *Drosophila* to date. Here, we show that the route of infection is key for *Wolbachia*-mediated antibacterial protection. *Drosophila melanogaster* carrying *Wolbachia* showed reduced mortality during enteric—but not systemic—infection with the opportunist pathogen *Pseudomonas aeruginosa*. *Wolbachia*-mediated protection was more pronounced in male flies and is associated with increased early expression of the antimicrobial peptide *Attacin A*, and also increased expression of a reactive oxygen species detoxification gene (*Gst D8*). These results highlight that the route of infection is important for symbiont-mediated protection from infection, that *Wolbachia* can protect hosts by eliciting a combination of resistance and disease tolerance mechanisms, and that these effects are sexually dimorphic. We discuss the importance of using ecologically relevant routes of infection to gain a better understanding of symbiont-mediated protection.

1. Introduction

Beneficial microbial infections are common throughout the animal kingdom, with profound effects on host physiology, behaviour, ecology and evolution [1–3]. Bacterial endosymbionts of insects, for example, are known to manipulate host reproduction [4,5], to alter the host's acquisition of essential nutrients [1,6] and to provide protection from the deleterious effects of parasites and pathogens [7,8]. *Wolbachia pipientis*—a maternally inherited, intracellular bacterium of arthropods and nematodes—is one of the best-studied microbial symbionts [9,10]. Its host range is vast, with recent estimates that 48–57% of all terrestrial arthropods [11], and at least 10% of all *Drosophila* species carry *Wolbachia* [12].

The ability of some *Wolbachia* strains to protect insect hosts from pathogenic infections makes it particularly relevant for potential bio-control of insect-vectored zoonotic infections, and more broadly relevant as modifiers of host ecology and mediators of pathogen-mediated selection in insects [9,10,13]. *Aedes aegypti* and *Aedes albopictus* mosquitoes, for example, have been shown to become more resistant to Dengue and Chikungunya viruses, as well as malaria-causing *Plasmodium* when they are experimentally transinfected with *Wolbachia* [14–16]. In *Drosophila*, there is also strong evidence that flies carrying *Wolbachia* are better able to survive infection by a number of RNA viruses [7,8].

In contrast with the strong evidence for *Wolbachia*-mediated protection from viral infections and being able to protect mosquitoes from bacterial challenge [16], its ability to protect its native fruit fly hosts from bacterial infections has not been clearly demonstrated [17,18]. In one study, *Wolbachia* did not affect the survival or immune activity of *Drosophila simulans* or *D. melanogaster* during systemic infection with *Pseudomonas aeruginosa*, *Serratia marcescens* or *Erwinia carotovora* [18], while another study found that the presence of *Wolbachia* had no effect on the ability to suppress pathogen growth during systemic infections by intracellular (*Listeria monocytogenes*, *Salmonella typhimurium*) or extracellular bacterial pathogens (*Providencia rettgeri*) [17]. Given that *Wolbachia* can provide broad-spectrum protection to mosquitoes against a range of pathogens, including bacteria [19], the lack of evidence for antibacterial protection in flies is puzzling.

Here, we show that the route of infection is key for *Wolbachia*-mediated protection in *Drosophila*, which we find to occur during enteric—but not systemic—infection by the opportunist pathogen *P. aeruginosa*. We exposed flies that were naturally infected with *Wolbachia*, and identical derived flies that were cured of *Wolbachia* infection, to *P. aeruginosa* either through intra-thoracic pricking (causing a systemic infection) or through the oral route of infection by feeding (causing an enteric infection). We monitored how within-host microbe loads and survival varied throughout the course of an enteric infection to assess if *Wolbachia*-mediated protection was due to differences in the bacterial clearance rate (resistance) or if it aided host survival in the presence of high microbe loads (tolerance); we also examined how these protective effects differed between male and female flies. We further characterized the expression of immune and damage repair genes previously shown to be involved in enteric bacterial infection in *Drosophila*.

2. Material and methods

(a) Fly stocks

Experiments were carried out using long-term laboratory stocks of *D. melanogaster* Oregon R (OreR). This line was originally infected with *Wolbachia* strain wMel (OreR^{Wol+}). To obtain a *Wolbachia*-free line of the same genetic background (OreR^{Wol-}), OreR^{Wol+} flies were cured of *Wolbachia* by rearing them on cornmeal Lewis medium supplemented with 0.05 mg ml^{-1} tetracycline. This treatment was carried out at least 3 years before these experiments were conducted, and the *Wolbachia* status of both fly lines was verified using PCR with primers specific to *Wolbachia* surface protein (*wsp*): forward (5′–3′): GTCCAATAGCTGATGAAGAA AC; reverse (5′–3′): CTGCACCAATAGCGCTATAAA. Both lines were kept as long-term laboratory stocks on a standard diet of cornmeal Lewis medium, at a constant temperature of 18 ± 1°C with a 12 L : 12 D cycle. Prior to the experiment, fly lines were raised on Lewis food at 25°C, with a 12 L : 12 D cycle for at least two generations. To standardize the larval density of experimental flies, replicate vials were set up containing ten, 2- to 4-day-old mated females from each OreR^{Wol-} or OreR^{Wol+} fly line who were left to lay eggs for 48 h to ensure that larval densities were comparable across all replicates, and that offspring were age-matched (within 48 h). Maternal flies from each line were sampled from at least four different bottles in order to avoid potential confounding effects of bottle-specific differences in fly microbiota.

(b) Bacterial cultures

Pseudomonas aeruginosa is a common Gram-negative bacterium with a broad host range, infecting insects, nematodes, plants and vertebrates, and is found in most environments [20,21]. Enteric infection of *Drosophila* by *P. aeruginosa* results in pathology to intestinal epithelia due to the formation of a bacterial biofilm in the crop, a food storage organ in the foregut [22,23]. In most enteric infections, *P. aeruginosa* growth is restricted to the crop, and is sufficient to cause death [22,24]. Infections were carried out using the *P. aeruginosa* reference strain PA14, which has been shown to have a very broad host range [25,26]. To obtain isogenic PA14 cultures, a frozen stock culture was streaked onto fresh LB agar plates and single colonies were inoculated into 50 ml LB broth and incubated overnight at 37°C with shaking at 150 r.p.m. Overnight cultures were diluted 1 : 100 into 500 ml fresh LB broth and incubated again at 37°C with shaking at 150 r.p.m. At the mid-log phase ($OD_{600} = 1.0$), we harvested the bacterial cells by centrifugation at 8000 r.p.m. for 10 min, washed the cells twice with 1 × PBS and re-suspended the bacterial pellet in 5% sucrose. The final inoculum was adjusted to $OD_{600} = 25$, and this was the bacterial inoculum used for all flies inoculated orally (enteric infection).

(c) Enteric and systemic bacterial infection

For systemic infection, flies were pricked in the pleural suture with a needle dipped in a mid-log phase ($OD_{600} = 1.0$) PA14 culture. Control flies were pricked with a needle dipped in sterile LB broth. For oral exposure (enteric infection), a concentrated PA14 inoculum ($OD_{600} = 25$) was spotted onto a sterile filter paper (80 µl per filter paper) and placed onto a drop of solidified 5% sugar agar inside the lid of a 7 ml Bijou tube. For the uninfected control treatment, filters received the equivalent volume of 5% sucrose solution only. Two- to 4-day-old flies were sex sorted and transferred individually to empty plastic vials: 180 (90 male and 90 female) OreR^{Wol+}, and 180 (90 male and 90 female) OreR^{Wol-}. Following 2–4 h of starvation, flies were transferred individually to 7 ml Bijou tubes, and covered with previously prepared lids containing a filter paper soaked in PA14 culture. Flies were left to feed on the bacterial culture for approximately 12 h at 25°C. Following this period, we sacrificed six exposed and two control flies and counted CFUs by plating the fly homogenate in *Pseudomonas* isolating media (PAIM). The remaining flies were transferred to vials containing 5% sugar agar and incubated at 25°C.

(d) Survival assays

We carried out separate experiments to measure how the presence of *Wolbachia* affected fly mortality during either enteric or systemic infection, with identical fly rearing and bacterial cultural conditions as those described above. For each survival assay (enteric or systemic infection routes), 2- to 4-day-old flies were sexed and exposed in groups of 10 flies to PA14, as described above. For each combination of male or female OreR^{Wol+} and OreR^{Wol-} line, we set up 10 flies per 10 replicates vials. Flies that died from infection were recorded every hour until all flies had died (systemic infection), or every 24 h for up to 8 days (enteric infection).

(e) Quantification of within-host bacterial loads in orally infected flies

Following the initial 12 h exposure, every 24 h, we randomly sampled five to seven live flies per sex and *Wolbachia* status and quantified the microbe loads present inside the flies. Briefly, a single fly was removed from the vial and transferred to 1.5 ml microcentrifuge tubes. To guarantee we were only quantifying CFUs present inside the fly, and not those possibly on its surface, each fly was surface sterilized by adding 75% ethanol for 30–60 s to kill the outer surface bacterial species. Ethanol was discarded and flies were washed twice with distilled water. Plating 100 µl of the second wash in LB agar confirmed this method was

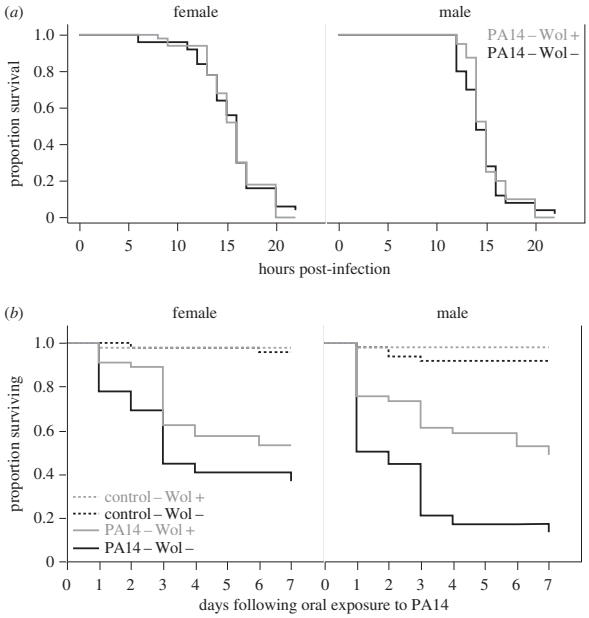


Figure 1. Fly survival after systemic oral infection with *P. aeruginosa* PA14. $OreR^{Wol-}$ (black) and $OreR^{Wol+}$ (grey) were either (*a*) pricked with a needle dipped in PA14 culture (OD = 1), or (*b*) left to feed on a PA14 culture (OD = 25) or on a control solution of 5% sugar for 12 h. Survival was monitored for 24 h (systemic infection) or daily (oral infection). In systemic infections, 100% of control flies survived over the 24 h period. In orally exposed flies, control flies are shown as dotted lines. Each data point shown is the mean of 10 replicate groups of 10 flies; these data were analysed using a Cox proportional hazard model.

efficient in cleaning the surface of the fly (no viable CFUs were detected). Each washed whole fly was placed in 1 ml of 1× PBS in a 1.5 ml screw-top microcentrifuge tube, centrifuged at 5000 r.p.m. for 1 min and the supernatant was discarded. Two hundred microlitres of LB broth were then added to each tube and the flies were thoroughly homogenized using a motorized pestle for 1 min. A 100 µl aliquot of homogenate was taken for serial dilution and different dilutions were plated on PAIM agar plates, incubated at 37°C for 24–48 h and viable CFUs were counted.

(f) Statistical analyses of host survival and microbe loads and tolerance

Fly survival was analysed using a Cox proportional hazards model to compare survival rates, with fly 'sex', 'infection status' and '*Wolbachia* status' and their interactions as fixed effects. The significance of the effects was assessed using likelihood ratio tests following a χ^2-distribution. For flies that were exposed orally to PA14, we compared between pairs of treatments (control versus infected or with and without *Wolbachia*) using the Cox risk ratios. In orally infected flies, changes in the bacterial load within-hosts were analysed with a linear model with $\log_{10}$CFU as the response variable, and fly 'sex', '*Wolbachia* status' and 'time (DPI)' as a continuous covariate. To assess sex- and *Wolbachia*-mediated differences in how sick a fly gets for a given pathogen load (tolerance), for each time point, we took the survival probability (as a measure host health) and PA14 CFUs present within the flies (as a measure of microbe load) for five replicate flies in each sex/*Wolbachia* combination, and fitted a four-parameter logidsitic model to this relationship [27] (see the electronic supplementary material, table S1 and accompanying text for details). All analyses were conducted in JMP 12 (SAS). Full model output tables can be found in electronic supplementary material, tables S1–S7.

(g) Gene expression

We tested for differences in the expression of genes known to be involved in either bacterial clearance (*PGRP-LC*, *PGRP-LE*, *attacin A*) or in the response to stress and gut damage (*gstD8*, *gadd45*, *CG32302*) during enteric bacterial infection [28–30] using qRT–PCR. Details on specific genes are given in the main text. Gene-specific primers are reported in the electronic supplementary material, table S2 and PCR conditions are reported in the electronic supplementary material.

3. Results

(a) Flies carrying *Wolbachia* show reduced mortality during enteric but not systemic bacterial infection

All flies infected systemically with PA14 by intra-thoracic pricking died within 24 h (figure 1*a*), and in line with previous work [18], we did not detect any significant effect of *Wolbachia* status on the rate at which these flies died (Cox proportional hazard model, likelihood ratio $\chi^2 = 0.003$, d.f. = 1, $p = 0.959$), or any effect of sex ('sex' effect, $\chi^2 = 0.860$, d.f. = 1, $p = 0.354$); 100% of control flies (pricked with sterile LB broth) survived during the same period. Flies that ingested and acquired an enteric infection of PA14 died at a faster

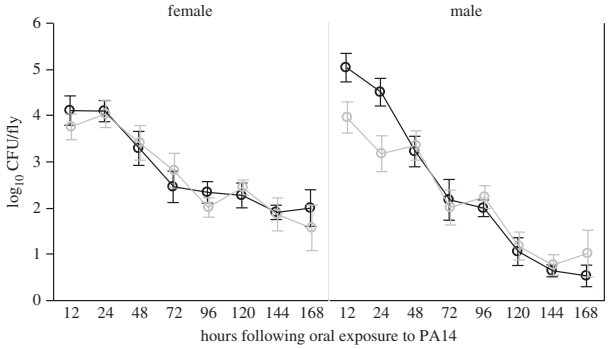


Figure 2. Within-host microbe loads. The number of viable within-host CFUs was quantified in five to seven individual live flies following 12 h of oral exposure, and then every 24 h for a week. Males and females are plotted separately for $OreR^{Wol-}$ (black) and $OreR^{Wol+}$ (grey) flies. Data shown are means ± s.e.m.

rate than control flies exposed only to a sucrose solution (figure 1*b*; 'infection status' effect, likelihood ratio $\chi^2 = 64.27$, d.f. = 1, $p < 0.0001$). Fly mortality during enteric infection was significantly affected by their *Wolbachia* status, but the extent of protection depended on fly sex (*Wolbachia* status × sex interaction $\chi^2 = 8.50$, d.f. = 1, $p = 0.0036$). This protective effect was not significant in female flies: the Cox risk ratio showed that females without *Wolbachia* were 1.58 more likely to die than infected females carrying *Wolbachia* (pairwise contrast: $p = 0.06$). The protection in male flies was more pronounced, as not carrying *Wolbachia* made PA14-infected males 2.26 times more likely to die than their infected *Wolbachia*-positive counterparts (pairwise contrast, $p < 0.001$; figure 1*b*).

(b) The presence of *Wolbachia* affects initial bacterial clearance in males but not in females during enteric infection

To understand the cause of the increased survival during enteric but not systemic infection protection, we focused on flies that acquired infection orally. Bacterial loads decreased over the course of the experiment in both male and female flies (figure 2) time effect ($F_{7,186} = 48.81$, $p < 0.0001$). We detected a significant statistical interaction between *Wolbachia* status, time and sex (electronic supplementary material, table S4), suggesting that the effects of *Wolbachia* on the rate of bacterial clearance are sex-specific. This was confirmed in a separate analysis for each sex: in females, there was no effect of *Wolbachia* on the rate at which PA14 was cleared (*Wolbachia* status × time interaction $F_{1,103} = 0.032$, $p = 0.858$), while in males, there was a significant effect of *Wolbachia* on how microbe loads changed with time (*Wolbachia* status × time interaction $F_{1,103} = 9.28$, $p = 0.003$). This effect is reflected in the difference in within-host CFUs measured at 12 and 24 h post-infection, where male flies harbouring *Wolbachia* showed 10-fold lower microbe loads compared with those without *Wolbachia* (figure 2; Wol+: 3.86 ± 0.22 $\log_{10}$ CFU; Wol−: 4.56 ± 0.22 $\log_{10}$ CFU; $F_{1,20} = 5.27$, $p = 0.033$). While we detected significant sex-specific effects of *Wolbachia* status on feeding (see the electronic supplementary material for feeding assay details and table S3 and figure S1), they were not consistent with changes in microbe loads, which were higher in *Wolbachia*-positive males.

(c) The presence of *Wolbachia* changes the disease tolerance profile of male flies

Independently of *Wolbachia* status, we observed that males and females showed different patterns of bacterial clearance over time (figure 2; electronic supplementary material, table S4 'time × sex' interaction). While males appeared to be able to clear the infection almost entirely within a week (mean ± s.e.m. 0.85 ± 0.29 $\log_{10}$ CFU per fly at 168 h post-exposure), females appeared to stop clearing infection after 96 h, maintaining a relatively stable bacterial load of about 100 CFUs per fly until the end of the experiment (figure 2). While we might expect this to result in higher female mortality, female flies showed similar survival to males following gut infection (figure 1*b*). Male flies, however, experienced increased survival when they were *Wolbachia*-positive compared with *Wolbachia*-negative males (figure 1*b*), even though the rate at which both groups clear infection appear identical (figure 2). This suggests that males benefit from increased infection tolerance in the presence of *Wolbachia*.

To better assess these differences in disease tolerance, we analysed the relationship between host health and microbe load for matching time-points (see the electronic supplementary material for details on analysis of disease tolerance; figure 3). In all cases, a nonlinear four-parameter logistic model described these data better than a linear model (electronic supplementary material, table S1). In female flies, the logistic model explained one-quarter of the variance ($R^2 = 0.24$), and a formal parallelism test found that the curves did not show significantly different shapes according to *Wolbachia* infection status ($F_{3,72} = 0.886$, $p = 0.452$). In male flies, the four-parameter logistic model explained over half the total variance ($R^2 = 0.57$), and a formal parallelism test revealed significant differences in the shapes of these two tolerance curves between *Wolbachia*-positive and *Wolbachia*-negative males ($F_{3,72} = 2.98$, $p = 0.037$). These differences arise not only to the consistently lower maximum and baseline survival in *Wolbachia*-negative males regardless of microbe load (figure 3), but also due to differences in the inflection point of each curve which occurs later in the infectious period in *Wolbachia*-positive male flies (figure 3).

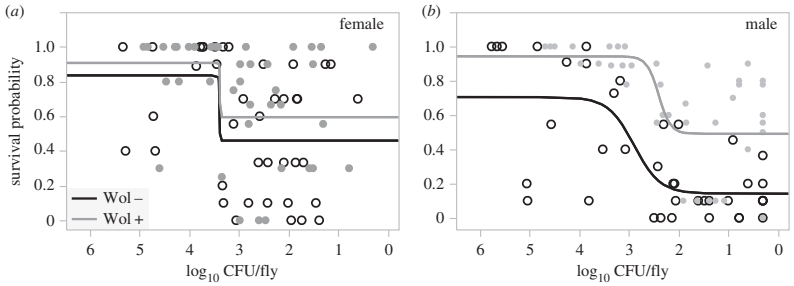


Figure 3. Disease tolerance. To measure disease tolerance, we analysed the relationship between host health and microbe loads. For each time point, we plot the survival probability (as a measure of health) against the microbe load (number of CFU per fly) for five biological replicates per sex and *Wolbachia* combination. Here, we show the fit of a four-parameter logistic model to the data (see electronic supplementary material, table S1 for model fits, and accompanying text for analysis details). The *x*-axis is reversed to read from beginning to the end of the infection (only clearance occurred).

(d) *Wolbachia*-positive flies show increased expression of immune deficiency pathway genes during the early stages of enteric infection

The immune deficiency (IMD) pathway is known to play an active role in the response to enteric bacterial infection [28,29]. We therefore tested whether flies carrying *Wolbachia* showed increased expression of genes involved in IMD-mediated antimicrobial immunity. Specifically, we measured the expression of genes that have been previously shown to be upregulated during enteric bacterial infection in *D. melanogaster* [28]: PGRP-LC, a peptidoglycan trans-synaptic signalling molecule that acts as a pattern recognition molecule in the anterior fly midgut [29]; PGRP-LE, an intracellular peptidoglycan that is especially active in the posterior midgut [29]; and Attacin A, an antimicrobial peptide (AMP) that is triggered by the IMD pathway during infection by Gram-negative bacteria [30]. In all genes, we detected significant time-dependent effects of *Wolbachia* status, and for the expression Attacin A, we also detected sex-dependent effects of *Wolbachia* carriage (see electronic supplementary material, table S6; figure 4); for these significant interactions, we report the relevant pairwise contrasts. In *Wolbachia*-positive females, we observed a significant increase in expression relative to uninfected females of PGRP-LC (figure 4*a*, $p = 0.0002$) and PGRP-LE (figure 4*b*, $p = 0.004$) at 96 h post-infection. Overall, there was no effect of *Wolbachia* on the expression of either receptor gene in male flies, but we observed a significant three- to fourfold increase in the expression of the AMP Attacin A in *Wolbachia*-positive males at both 24 h ($p = 0.002$) and 96 h ($p < 0.001$) post-infection (figure 4*c*).

(e) *Wolbachia* is associated with higher expression of the reactive oxygen species detoxification gene *gstD8* in males during enteric infection

We hypothesized that in addition to the antimicrobial activity of Attacin A, mechanisms involved in detoxifying reactive oxygen species (ROS), commonly produced during enteric infection with PA14 [31], could also underlie the differences in survival between flies with and without *Wolbachia* (figure 1). The expression of GstD8—involved in ROS detoxification [31]—showed significant sex-specific effects of *Wolbachia* carriage over time during enteric infection with PA14 (electronic supplementary material, table S6). GstD8 expression was significantly higher at 96 h post-infection in males harbouring *Wolbachia* compared with those without the symbiont (figure 4*d*, $p = 0.001$), while no difference was observed in female GstD8 expression according to *Wolbachia* status ($p = 0.08$, figure 4*d*).

(f) *Wolbachia* is associated with higher expression of epithelial repair genes in females during enteric infection

Oral infection often results in damage to insect guts [29], so we also measured the expression of genes involved in tissue damage repair (*gadd45*) and a component of the peritrophic matrix (*CG32302*) [28]. Both genes showed sex-specific effects of *Wolbachia* carriage that changed over time (electronic supplementary material, table S7). *Gadd45* expression was marginally higher in *Wolbachia*-positive females compared with those without *Wolbachia* at 96 h post-infection (figure 4*e*, $p < 0.001$). CG32302 expression was only transiently differentially expressed in *Wolbachia*-positive females at 24 h post-infection (figure 4*f*, $p = 0.01$), but not at the other time-points. *Wolbachia*-negative males showed a significantly higher expression relative to *Wolbachia*-positive males of both *gadd45* (figure 4*e*, $p = 0.02$) and CG32302 (figure 4*f*, $p = 0.01$) at 24 h post-infection, although this difference was no longer observed by 96 h post-infection.

4. Discussion

Wolbachia plays a key role in conferring protection from pathogens in their insect hosts [7,8]. In its natural host *Drosophila*, *Wolbachia*-mediated protection is especially evident during viral infections, but protection from bacterial pathogens in *Drosophila* had not been demonstrated to date. Here, we provide strong evidence that the route of infection is important for *Wolbachia*-mediated protection from bacterial infection. We find that *Wolbachia* can protect *Drosophila* from enteric bacterial infection by eliciting a combination of antimicrobial and damage repair mechanisms, and that these protective effects are sexually dimorphic.

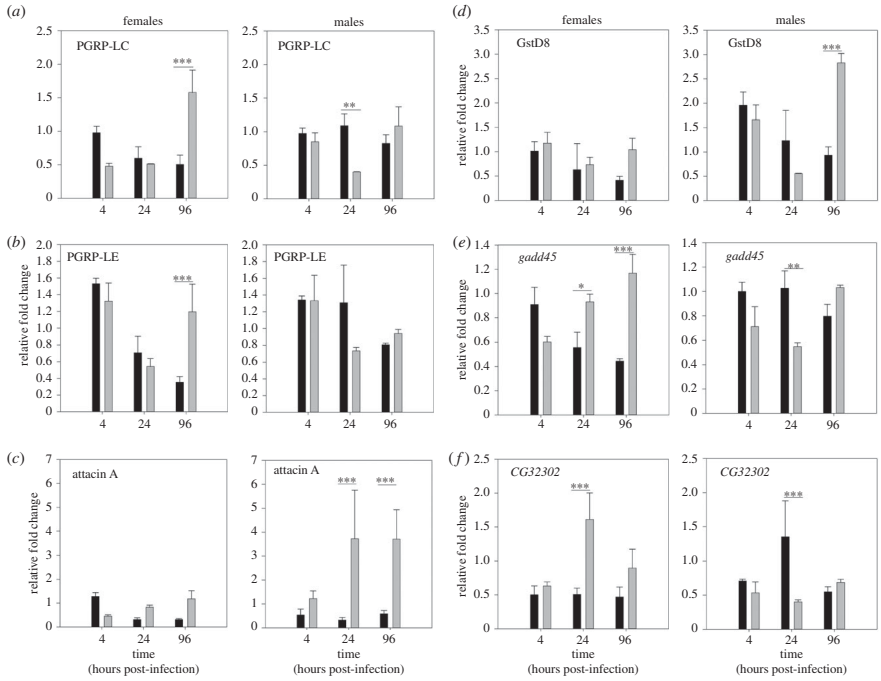


Figure 4. Gene expression relative to *rp49* control gene in infected flies relative to uninfected flies. The expression of genes involved in IMD-mediated antimicrobial immunity were measured: (*a*) PGRP-LC, a peptidoglycan pattern recognition molecule in the anterior fly midgut; (*b*) PGRP-LE, an intracellular peptidoglycan active in the posterior midgut; and (*c*) Attacin A, an AMP activated during infection by Gram-negative bacteria. We also measured the expression of GstD8—involved in ROS detoxification (*d*) and other genes involved in tissue damage repair (*gadd45*) (*e*) as well as and a component of the peritrophic matrix (*CG32302*) (*f*). *Wolbachia*-positive flies are shown in grey, and *Wolbachia*-negative flies in black. Data show the mean ± s.e. of pooled technical duplicates for three biological groups of five flies for each sex/*Wolbachia* combination, exposed orally to *P. aeruginosa* infection.

(a) The route of infection matters for *Wolbachia* protection

The role of *Wolbachia* in protecting hosts from infection, either by increasing resistance or tolerance, is known in *Drosophila*–virus interactions, but previous work testing for antibacterial protection in *Drosophila* did not find a significant effect of *Wolbachia* [17,18]. Typically, flies in previous studies were inoculated by intra-thoracic pricking or injection, and therefore experienced a systemic infection. In the wild, however, infections are more likely to be acquired through the faecal–oral route (during feeding on decomposing fruit), with most pathogens colonizing the gut before being shed through the faeces. *Drosophila*–*Wolbachia* interactions would therefore have co-evolved mainly under selection by pathogen infection in the gut, and any antibacterial protection that may have evolved as a consequence would not be expected to manifest during a highly virulent systemic infection [30,32]. Further, if *Wolbachia*-mediated protection is especially efficient in the fly gut, the damage caused by a generalized systemic infection could overwhelm any localized protection by *Wolbachia*, which could explain the lack of observed protection in previous studies of systemic bacterial infection in *Drosophila*.

Work in a number of insect host species, including flies [32,33], moths [34] and aphids [35], has highlighted how the route of infection can affect the progression and the outcome of disease due to differences in the mortality and the dynamics of pathogen growth. Distinct immune pathways are also elicited during systemic and enteric infection; recent work has shown that in *Drosophila*, the Toll-Dorsal pathway is required to defend from gut infection but not systemic infection by Drosophila C virus [36]. In addition to affecting the outcome of an infection at the individual level, these differences and immune deployment and disease outcome may even have more profound consequences for how hosts evolve in response to pathogens [32]. Studies of host resistance and tolerance should therefore favour natural routes of infection in order to gain a more realistic understanding of the mechanisms that hosts have evolved to fight infection.

(b) *Wolbachia*-mediated protection is a combination of pathogen clearance and damage limitation

The mechanisms underlying *Wolbachia*-mediated protection are largely unclear, especially given that the extent of the protection and whether it acts to increase resistance or tolerance

appear to be pathogen-specific [7,37,38]. In mosquitoes, *Wolbachia* protection appears to be involved in a combination of general immune priming [39], resource competition between *Wolbachia* and infectious agents [40], and the regulation of host genes involved in blocking pathogen replication [41]. However, mosquitoes have only been recently transinfected with *Wolbachia* and it is unclear if we might expect the same mechanisms to underlie protection in *Drosophila* which has a long coevolutionary history with *Wolbachia*. In *Drosophila*, *Wolbachia*-mediated antiviral protection is variable among strains of *Wolbachia* and correlates strongly with the reduction in viral titres within hosts [38], suggesting that *Wolbachia* generally enhances the ability to clear pathogens (increasing host resistance). These results contrast with work showing that *D. simulans* infected with *Wolbachia* strain wAu can withstand high virus titres without high levels of mortality [42], indicating that *Wolbachia* can, in some cases, also promote disease tolerance. Notably, *Drosophila*–*Wolbachia* associations that confer antiviral protection following systemic viral infection have also been found to protect adult flies following oral exposure to Drosophila C virus, although this was but not observed when flies were challenged as larvae [43].

Bacterial loads did not increase throughout the course of the infection, but were cleared at a near exponential rate (figure 2). Despite this, flies still died from infection, although the presence of *Wolbachia* was associated with a reduction in initial microbe loads and lower mortality in male flies, as well as an increase in the expression of the AMP Attacin A. One possibility is that most of the damage experienced by the host happens at the early stages of infection, as the greatest difference in male mortality happens within the first 48 h when bacterial loads are on average 10 times higher in *Wolbachia*-negative flies. It is therefore possible that the increased expression of *Attacin A* within the first 96 h post-infection (figure 4) may have led to the lower bacterial loads observed in the early stages of infection (figure 2), therefore minimizing gut damage caused by pathogen growth.

Given that we observed *Wolbachia*-associated changes in the tolerance profiles of male flies, we also chose to measure the expression of genes involved in damage repair. We investigated the expression of *gstD8*, involved in ROS detoxification, because it was previously shown to be upregulated during enteric infection in *Drosophila* with another bacterial pathogen, *E. carotovora* [28]. We found that the expression of *gstD8* was elevated in *Wolbachia*-positive males, but not female flies, following 96 h of oral exposure to *P. aeruginosa*, which is consistent with the increased survival observed in *Wolbachia*-positive males compared with males without the endosymbiont (figure 1*b*).

In addition to this detoxification response, we also measured the expression of genes involved in tissue damage repair (*gadd45*) and a component of the peritrophic matrix (*CG32302*). In males, the presence of *Wolbachia* did not result in an increase in these genes within 96 h of oral exposure to PA14, but females carrying the endosymbiont showed significantly higher expression than *Wolbachia*-negative flies of *gadd45*. This may indicate that *Wolbachia* could induce different damage limitation mechanisms in males and females. We also observed transient increases in the expression of CG32302, another component of gut renewal, in *Wolbachia*-positive females at 24 h post-infection. There was also a transient increase in expression at 24 h post-infection of *gadd45* and *CG32302* in *Wolbachia*-negative males (figure 4). We interpret these increases as a response to increased damage to gut tissue cause by the 10-fold higher bacterial loads in these flies after 24 h (figure 2), which was avoided in *Wolbachia*-positive males by *attacinA*-mediated clearance.

While previous work found no difference in genome-wide expression levels in adult *Drosophila* with or without *Wolbachia* [44], and only mild upregulation of immune genes has been reported in *Drosophila* cell lines that are transiently infected [45], our gene expression results indicate that *Wolbachia*-mediated protection from enteric bacterial infection relies on a combination of antimicrobial activity and damage repair mechanisms.

(c) Sex differences in immunity and *Wolbachia*-mediated protection

A clear result from our work is that males and females vary in their ability to clear (figure 2) and tolerate infection (figure 3). While males and females are generally susceptible to the same pathogens, sexual dimorphism in the immune response is apparent in a wide range of species [46–48], and is documented for all classes of viral, bacterial, fungal and parasitic infections (see [49] for review). In invertebrate hosts, and especially in *Drosophila*, most studies investigating the ability to resist or tolerate bacterial and viral infections have focused primarily on the underlying immune mechanisms [21,29,50–52]. Typically, these studies have not focused on sexual differences in these mechanisms (but see [53]). However, our results, together with a large body of work on immune sexual dimorphism [54], show that resistance and tolerance mechanisms are likely to vary between males and females. The causes of sex differences in immunity are not clear, but one likely source of variation is that many immune genes are linked to sex chromosomes [55] and so X-linked regulators of fly innate immunity could underlie the sexually dimorphic clearance and tolerance response that we observed.

Moreover, this sexual dimorphism was modified by the presence of *Wolbachia*. We found that the tolerance curves of *Wolbachia*-positive males were always higher than those without *Wolbachia*, indicating that the presence of the endosymbiont results in greater health throughout the infection. However, we did not observe the same level of protection in female flies (figures 1–3). It is also notable that the inflection point of the curve (indicating a severe decline in survival) occurs much later in the infection in *Wolbachia*-positive males (although it does occur eventually), and that the overall severity of these infections in reduced (the baseline of the curve is higher) in *Wolbachia*-positive males.

This outcome was unexpected because maternally inherited symbionts, such as *Wolbachia*, are well known to use specific adaptive strategies to spread and persist within insect populations, usually providing fitness benefits to female hosts. This makes the greater protection in males surprising. One possibility is that the level of protection we observe in females is in fact the best adaptive strategy for *Wolbachia*, especially if the mechanism of protection (an increase in the expression of AMPs in males) could also result in lower *Wolbachia* titres, and hence lower *Wolbachia* fitness. Therefore, a possible explanation for lower antibacterial protection in females is that *Wolbachia* evolution has resulted in a balance between the fitness benefits to *Wolbachia* of reduced host pathology against the fitness costs of reduced *Wolbachia* titre.

5. Concluding remarks

Together, our results show that *Wolbachia* can protect *Drosophila* from enteric bacterial infection by eliciting a combination of antimicrobial and disease tolerance mechanisms associated with an initial upregulation of antimicrobial activity, and that these protective effects are sexually dimorphic. Future studies of symbiont-mediated protection should therefore favour natural routes of infection in order to gain a more realistic picture of the mechanisms that hosts have evolved to fight infection.

Data accessibility. All data presented in this manuscript can be accessed on the Dryad Data Depository doi:10.5061/dryad.d0vp8 [56].

Authors' contributions. P.F.V. conceived the study. V.G., R.B.V., J.S.-J., K.M.M. and P.F.V. conducted experimental work. V.G. and P.F.V. analysed the data. P.F.V. wrote the manuscript. S.P.B. and P.F.V. contributed reagents and consumables. All authors commented on drafts of the manuscript.

Competing interests. We declare we have no competing interests.

Funding. This work was supported by a strategic award from the Wellcome Trust for the Centre for Immunity, Infection and Evolution (http://ciie.bio.ed.ac.uk; grant reference no. 095831), and by a Society in Science—Branco Weiss fellowship (http://www.society-in-science.org), both awarded to P.F.V. P.F.V. is also supported by a Chancellor's fellowship from the School of Biological Sciences, University of Edinburgh, UK.

Acknowledgements. We thank R. Popat for technical assistance, D. Obbard, F. Waldron, T. Little and S. Lewis and three reviewers for constructive suggestions that improved the manuscript, and H. Cowan and H. Borthwick for media preparation.

References

1. Douglas AE. 2015 Multiorganismal insects: diversity and function of resident microorganisms. *Annu. Rev. Entomol.* **60**, 17–34. (doi:10.1146/annurev-ento-010814-020822)
2. Gandon S, Vale PF. 2014 The evolution of resistance against good and bad infections. *J. Evol. Biol.* **27**, 303–312. (doi:10.1111/jeb.12291)
3. Bennett GM, Moran NA. 2015 Heritable symbiosis: the advantages and perils of an evolutionary rabbit hole. *Proc. Natl Acad. Sci. USA* **112**, 10 169–10 176. (doi:10.1073/pnas.1421388112)
4. Werren JH, Baldo L, Clark ME. 2008 *Wolbachia*: master manipulators of invertebrate biology. *Nat. Rev. Microbiol.* **6**, 741–751. (doi:10.1038/nrmicro1969)
5. Engelstädter J, Hurst GDD. 2009 The ecology and evolution of microbes that manipulate host reproduction. *Annu. Rev. Ecol. Evol. Syst.* **40**, 127–149. (doi:10.1146/annurev.ecolsys.110308.120206)
6. Douglas AE. 1998 Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria Buchnera. *Annu. Rev. Entomol.* **43**, 17–37. (doi:10.1146/annurev.ento.43.1.17)
7. Teixeira L, Ferreira Á, Ashburner M. 2008 The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biol.* **6**, e1000002. (doi:10.1371/journal.pbio.1000002)
8. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN. 2008 *Wolbachia* and virus protection in insects. *Science* **322**, 702. (doi:10.1126/science.1162418)
9. Brownlie JC, Johnson KN. 2009 Symbiont-mediated protection in insect hosts. *Trends Microbiol.* **17**, 348–354. (doi:10.1016/j.tim.2009.05.005)
10. Hamilton PT, Perlman SJ. 2013 Host defense via symbiosis in *Drosophila*. *PLoS Pathog.* **9**, e1003808. (doi:10.1371/journal.ppat.1003808)
11. Weinert LA, Araujo-Jnr EV, Ahmed MZ, Welch JJ. 2015 The incidence of bacterial endosymbionts in terrestrial arthropods. *Proc. R. Soc. B* **282**, 20150249. (doi:10.1098/rspb.2015.0249)
12. Mateos M, Castrezana SJ, Nankivell BJ, Estes AM, Markow TA, Moran NA. 2006 Heritable endosymbionts of *Drosophila*. *Genetics* **174**, 363–376. (doi:10.1534/genetics.106.058818)
13. Johnson KN. 2015 Bacteria and antiviral immunity in insects. *Curr. Opin. Insect Sci.* **8**, 97–103.
14. Moreira LA *et al.* 2009 A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, chikungunya, and plasmodium. *Cell* **139**, 1268–1278. (doi:10.1016/j.cell.2009.11.042)
15. Bian G, Xu Y, Lu P, Xie Y, Xi Z. 2010 The endosymbiotic bacterium *Wolbachia* induces resistance to dengue virus in *Aedes aegypti*. *PLoS Pathog.* **6**, e1000833. (doi:10.1371/journal.ppat.1000833)
16. Kambris Z, Blagborough AM, Pinto SB, Blagrove MSC, Godfray HCJ, Sinden RE, Sinkins SP. 2010 *Wolbachia* stimulates immune gene expression and inhibits plasmodium development in *Anopheles gambiae*. *PLoS Pathog.* **6**, e1001143. (doi:10.1371/journal.ppat.1001143)
17. Rottschaefer SM, Lazzaro BP. 2012 No effect of *Wolbachia* on resistance to intracellular infection by pathogenic bacteria in *Drosophila melanogaster*. *PLoS ONE* **7**, e40500. (doi:10.1371/journal.pone.0040500)
18. Wong ZS, Hedges LM, Brownlie JC, Johnson KN. 2011 *Wolbachia*-mediated antibacterial protection and immune gene regulation in *Drosophila*. *PLoS ONE* **6**, e25430. (doi:10.1371/journal.pone.0025430)
19. Ye YH, Woolfit M, Rancès E, O'Neill SL, McGraw EA. 2013 *Wolbachia*-associated bacterial protection in the mosquito *Aedes aegypti*. *PLoS Negl. Trop. Dis.* **7**, e2362. (doi:10.1371/journal.pntd.0002362)
20. Apidianakis Y, Rahme LG. 2009 *Drosophila melanogaster* as a model host for studying *Pseudomonas aeruginosa* infection. *Nat. Protoc.* **4**, 1285–1294. (doi:10.1038/nprot.2009.124)
21. Neyen C, Bretscher AJ, Binggeli O, Lemaitre B. 2014 Methods to study *Drosophila* immunity. *Methods San Diego Calif.* **68**, 116–128. (doi:10.1016/j.ymeth.2014.02.023)
22. Sibley CD, Duan K, Fischer C, Parkins MD, Storey DG, Rabin HR, Surette MG. 2008 Discerning the complexity of community interactions using a *Drosophila* model of polymicrobial infections. *PLoS Pathog.* **4**, e1000184. (doi:10.1371/journal.ppat.1000184)
23. Mulcahy H, Sibley CD, Surette MG, Lewenza S. 2011 *Drosophila melanogaster* as an animal model for the study of *Pseudomonas aeruginosa* biofilm infections *in vivo*. *PLoS Pathog.* **7**, e1002299. (doi:10.1371/journal.ppat.1002299)
24. Chugani SA, Whiteley M, Lee KM, D'Argenio D, Manoil C, Greenberg EP. 2001 QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. *Proc. Natl Acad. Sci. USA* **98**, 2752–2757. (doi:10.1073/pnas.051624298)
25. He J *et al.* 2004 The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. *Proc. Natl Acad. Sci. USA* **101**, 2530–2535.
26. Mikkelsen H, McMullan R, Filloux A. 2011 The *Pseudomonas aeruginosa* reference strain PA14 displays increased virulence due to a mutation in ladS. *PLoS ONE* **6**, e0029113. (doi:10.1371/journal.pone.0029113)
27. Gupta V, Vale PF. In press. Non-linear disease tolerance curves reveal distinct components of host responses to viral infection. *R. Soc. open sci.*
28. Buchon N, Broderick NA, Poidevin M, Pradervand S, Lemaitre B. 2009 Drosophila intestinal response to bacterial infection: activation of host defense and stem cell proliferation. *Cell Host Microbe* **5**, 200–211. (doi:10.1016/j.chom.2009.01.003)
29. Buchon N, Silverman N, Cherry S. 2014 Immunity in *Drosophila melanogaster*—from microbial recognition to whole-organism physiology. *Nat. Rev. Immunol.* **14**, 796–810. (doi:10.1038/nri3763)
30. Liehl P, Blight M, Vodovar N, Boccard F, Lemaitre B. 2006 Prevalence of local immune response against oral infection in a *Drosophila*/*Pseudomonas* infection model. *PLoS Pathog.* **2**, e56. (doi:10.1371/journal.ppat.0020056)
31. Ha E-M, Oh C-T, Bae YS, Lee W-J. 2005 A direct role for dual oxidase in *Drosophila* gut immunity. *Science* **310**, 847–850. (doi:10.1126/science.1117311)
32. Martins NE, Faria VG, Teixeira L, Magalhães S, Sucena É. 2013 Host adaptation is contingent upon the

infection route taken by pathogens. *PLoS Pathog.* **9**, e1003601. (doi:10.1371/journal.ppat.1003601)
33. Chambers MC, Jacobson E, Khalil S, Lazzaro BP. 2014 Thorax injury lowers resistance to infection in *Drosophila melanogaster*. *Infect. Immun.* **82**, 4380–4389. (doi:10.1128/IAI.02415-14)
34. Saejeng A, Siva-Jothy MT, Boots M. 2011 Low cost antiviral activity of *Plodia interpunctella* haemolymph *in vivo* demonstrated by dose dependent infection. *J. Insect Physiol.* **57**, 246–250. (doi:10.1016/j.jinsphys.2010.10.005)
35. Grenier A-M, Duport G, Pagès S, Condemine G, Rahbé Y. 2006 The phytopathogen *Dickeya dadantii* (Erwinia chrysanthemi 3937) is a pathogen of the pea aphid. *Appl. Environ. Microbiol.* **72**, 1956–1965. (doi:10.1128/AEM.72.3.1956-1965.2006)
36. Ferreira ÁG, Naylor H, Esteves SS, Pais IS, Martins NE, Teixeira L. 2014 The Toll-Dorsal pathway is required for resistance to viral oral infection in *Drosophila*. *PLoS Pathog.* **10**, e1004507. (doi:10.1371/journal.ppat.1004507)
37. Chrostek E, Marialva MSP, Esteves SS, Weinert LA, Martinez J, Jiggins FM, Teixeira L. 2013 *Wolbachia* variants induce differential protection to viruses in *Drosophila melanogaster*: a phenotypic and phylogenomic analysis. *PLoS Genet.* **9**, e1003896. (doi:10.1371/journal.pgen.1003896)
38. Martinez J, Longdon B, Bauer S, Chan Y-S, Miller WJ, Bourtzis K, Teixeira L, Jiggins FM. 2014 Symbionts commonly provide broad spectrum resistance to viruses in insects: a comparative analysis of *Wolbachia* strains. *PLoS Pathog.* **10**, e1004369. (doi:10.1371/journal.ppat.1004369)
39. Rancès E, Ye YH, Woolfit M, McGraw EA, O'Neill SL. 2012 The relative importance of innate immune priming in *Wolbachia*-mediated dengue interference. *PLoS Pathog.* **8**, e1002548. (doi:10.1371/journal.ppat.1002548)
40. Caragata EP, Rancès E, Hedges LM, Gofton AW, Johnson KN, O'Neill SL, McGraw EA. 2013 Dietary cholesterol modulates pathogen blocking by *Wolbachia*. *PLoS Pathog.* **9**, e1003459. (doi:10.1371/journal.ppat.1003459)
41. Zhang G, Hussain M, O'Neill SL, Asgari S. 2013 *Wolbachia* uses a host microRNA to regulate transcripts of a methyltransferase, contributing to dengue virus inhibition in *Aedes aegypti*. *Proc. Natl Acad. Sci. USA* **110**, 10 276–10 281. (doi:10.1073/pnas.1303603110)
42. Osborne SE, Leong YS, O'Neill SL, Johnson KN. 2009 Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. *PLoS Pathog.* **5**, e1000656. (doi:10.1371/journal.ppat.1000656)
43. Stevanovic AL, Arnold PA, Johnson KN. 2015 *Wolbachia*-mediated antiviral protection in *Drosophila* larvae and adults following oral infection. *Appl. Environ. Microbiol.* **81**, 8215–8223. (doi:10.1128/AEM.02841-15)
44. Teixeira L. 2012 Whole-genome expression profile analysis of *Drosophila melanogaster* immune responses. *Brief. Funct. Genomics* **11**, 375–386. (doi:10.1093/bfgp/els043)
45. Xi Z, Gavotte L, Xie Y, Dobson SL. 2008 Genome-wide analysis of the interaction between the endosymbiotic bacterium *Wolbachia* and its *Drosophila* host. *BMC Genomics* **9**, 1. (doi:10.1186/1471-2164-9-1)
46. Zuk M, McKean KA. 1996 Sex differences in parasite infections: patterns and processes. *Int. J. Parasitol.* **26**, 1009–1024. (doi:10.1016/S0020-7519(96)80001-4)
47. Marriott I, Huet-Hudson YM. 2006 Sexual dimorphism in innate immune responses to infectious organisms. *Immunol. Res.* **34**, 177–192. (doi:10.1385/IR:34:3:177)
48. Duneau D, Ebert D. 2012 Host sexual dimorphism and parasite adaptation. *PLoS Biol.* **10**, e1001271. (doi:10.1371/journal.pbio.1001271)
49. Cousineau SV, Alizon S. 2014 Parasite evolution in response to sex-based host heterogeneity in resistance and tolerance. *J. Evol. Biol.* **27**, 2753–2766. (doi:10.1111/jeb.12541)
50. Kemp C, Imler J-L. 2009 Antiviral immunity in *Drosophila*. *Curr. Opin. Immunol.* **21**, 3–9. (doi:10.1016/j.coi.2009.01.007)
51. Ayres JS, Schneider DS. 2012 Tolerance of infections. *Annu. Rev. Immunol.* **30**, 271–294. (doi:10.1146/annurev-immunol-020711-075030)
52. Schneider DS *et al.* 2007 *Drosophila* eiger mutants are sensitive to extracellular pathogens. *PLoS Pathog.* **3**, e41. (doi:10.1371/journal.ppat.0030041)
53. Vincent CM, Sharp NP. 2014 Sexual antagonism for resistance and tolerance to infection in *Drosophila melanogaster*. *Proc. R. Soc. B* **281**, 20140987. (doi:10.1098/rspb.2014.0987)
54. Nunn CL, Lindenfors P, Pursall ER, Rolff J. 2009 On sexual dimorphism in immune function. *Phil. Trans. R. Soc. B* **364**, 61–69. (doi:10.1098/rstb.2008.0148)
55. Hill-Burns EM, Clark AG. 2009 X-linked variation in immune response in *Drosophila melanogaster*. *Genetics* **183**, 1477–1491. (doi:10.1534/genetics.108.093971)
56. Gupta V, Vasanthakrishnan RB, Siva-Jothy J, Monteith KM, Brown SP, Vale PF. 2017 Data from: The route of infection determines *Wolbachia* antibacterial protection in *Drosophila*. Data Dryad Repository. (http://dx.doi.org/10.5061/dryad.d0vp8)

7.5.2 The Influence of Parasites on Insect Behavior.

Vale, P.F., Siva-Jothy, J., Morrill, A., Forbes M. (2018) The Influence of Parasites on Insect Behavior. In *Insect Behavior: from mechanisms to ecological and evolutionary consequences*. OUP Oxford. pp. 274-291

CHAPTER 18

The influence of parasites on insect behavior

Pedro F. Vale[1], Jonathon A. Siva-Jothy[1], André Morrill[2], and Mark R. Forbes[2]

[1]*Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, Scotland, UK*
[2]*Department of Biology, Carleton University, Ottawa, Canada*

18.1 Background

It is estimated that well over 50 per cent of extant species have a parasitic lifestyle, for at least some stage of their life cycle (Schmid-Hempel 2011). For example, parasitism is thought to have evolved at least once independently in ten of the twenty-one extant insect orders (Weinstein and Kuris 2016). It is perhaps not surprising that infection is widespread among host species from all taxa of life, and hosts have evolved a number of physiological, immunological, and behavioral responses to pathogens and parasites (Schmid-Hempel 2011). This chapter is concerned with behavioral responses to actual or potential infection by pathogens and parasites using insects as model hosts, although instances are highlighted where the players also include insect species that are parasites or parasitoids.

Insects, and their pathogens and parasites serve as ideal systems to investigate questions at the interface of infection and behavior. Questions such as how does behavior affect likelihood of infection? Or, is a novel or stereotypical behavior expressed following infection interpretable as a host adaptation, parasite adaptation, or by-product of infection not particularly beneficial to either the parasite or host individual? Insects are ideal hosts because we know much about disciplines related to infection and behavioral ecology, including insect natural history, genetics, physiology, immunology, and developmental biology. We generally know a great deal about insect biology because insects are important pests of agricultural crops (see Chapter 20), important vectors of some of the most deadly human diseases (see Chapter 21) and/or particularly amenable to laboratory rearing and protocols. Often our interpretations of insect behavioral response to infection rely on insight gleaned from those other disciplines. For example, consider that there are genes for behaviors and immune expression, and genetic variation in either could explain phenotypic variation in behavior. This is especially important if that phenotypic variation in behavior influences variation in infection risk or costs of infection.

Reviews are defined by the phenomena they include as well as those they ignore. In writing a chapter like this, there are literally hundreds of general observational and specific experimental studies from which to illustrate key concepts in the study of parasitism and host behavior, made much less daunting by the taxonomic focus on insects. We have focused further on some of the more iconic

Vale, P. F., Siva-Jothy, J. A., Morrill, A., and Forbes, M. R., *The influence of parasites on insect behaviour*. In: *Insect Behavior: From mechanisms to ecological and evolutionary consequences.* Edited by Alex Córdoba-Aguilar, Daniel González-Tokman, and Isaac González-Santoyo: Oxford University Press (2018).
DOI: 10.1093/oso/9780198797500.003.0018

and recent examples, where interpretations of insect behavioral responses to pathogens and parasites are fairly well understood because the systems are particularly amenable to investigation and interpretation. Although these tractable species associations for study may be seen as a non-random subset of nature, they provide examples of what evolved or plastic host responses are possible. One aspect that is largely ignored is what effect co-infecting pathogens and parasites have on insect behavioral responses to focal species. The fact that insect pathogens and parasites often have several possible host species for a given life history (infective) stage is also largely ignored (Rigaud et al. 2010). It is fully recognized that these aspects might prove important in determining the types or magnitudes of behavior expressed, but there is still much to discuss in the absence of treatment of these phenomena.

This chapter divides behavior conceptually into two broad categories—those that occur before infection possibly following encounter, or contact with pathogens and parasites or their cues, and, those behaviors that occur following infection (see Box 18.1a). Avoiding infection is the first line of defence and is known to occur in a broad range of taxa, being reasonably well-studied for insect hosts. The evidence that host insects detect and discriminate between clean and potentially infectious environments is reviewed, the importance of this avoidance for the consequences of infection, and there is also interest in whether avoidance behavior is context-dependent. A second broad line of inquiry concerns behavioral responses to infection. Behavioural 'responses' following infection are widely reported among animals, and can be classified more specifically into the following:

- **sickness behaviors** that benefit the host by conserving energetic resources during infection;
- parasitic manipulation that enhances parasite survival or transmission;
- a by-product of pathogenicity that does not necessarily benefit the host or the parasite.

The second section of this chapter reviews the evidence for adaptive 'sickness' behavior of hosts, whereas the third part of this chapter illustrates key concepts involved in the demonstration and consequences of parasitic manipulation of insect host behavior.

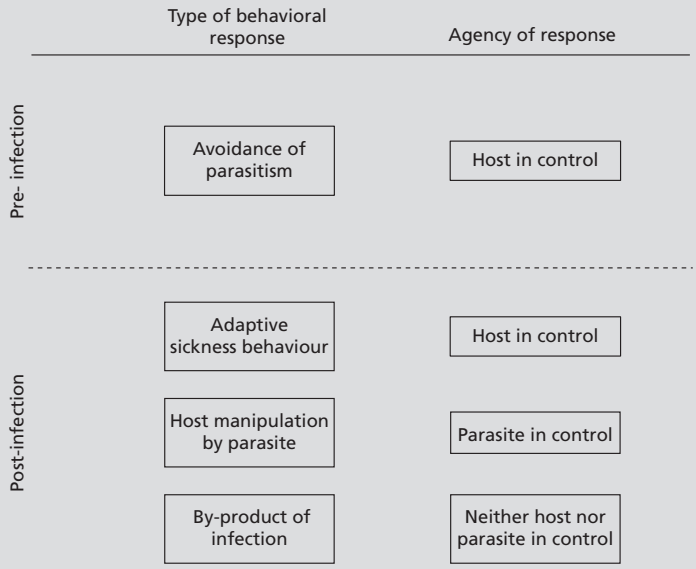
Some of the latest techniques in studying insect behavior in relation to infection are also discussed (Box 18.2). In addition to underscoring useful recording and computational techniques in the study and analysis of insect behavior, another common theme shared by the types of studies highlighted is the level of behavioral explanation being sought. Here, we rely on Tinbergen's (1963) four levels of explanation in behavior (Box 18.1b). Most of the work highlighted concerns the expected fitness value to the host or to the parasite of the host's behavioral response being investigated, and only some work concerns the mechanism or causation of behavioral change of infected insects. Box 18.3 on what is known about the neuroendocrine-immune axis in insects and its mechanistic relation to behavioral expression and immunity. Considerably less time will be spent on what is known about the evolutionary history and developmental trajectory of insect behavior in the context of infection, although both concepts are introduced briefly. The Chapter finishes by highlighting, for each section, tractable yet unresolved questions and issues whose consideration will help inform research for students of insect behavior in relation to infection by pathogens and parasites.

18.2 Infection avoidance behavior in insects

The first lines of host defence are **infection avoidance** behaviors that prevent parasites from infecting hosts. This **behavioral immunity** can be hugely effective because it obviously results in hosts avoiding the costs of parasite **virulence**, but also because hosts avoid potential negative consequences of immune deployment, such as immunopathology or costly investment in immune mechanisms (Curtis 2014). Avoidance behaviors rely on sensory systems detecting parasites or their cues, mechanisms that integrate this sensory information, and effector systems that, when activated, reduce host–parasite contact. This section discusses insect avoidance mechanisms with respect to the source of infection they avoid and/or the aspect of host ecology they affect.

Box 18.1 Guiding principles for the study of insect behaviour in relation to parasitism

Conceptual framework



Box 18.1 Categories of behavioural responses to parasitism with their timing in relation to infection and an indication of whether the host or the parasite is in control of the response.

Tinbergen's four levels of explanation

Nikolaas Tinbergen outlined four modes of questioning animal behaviors as a means of categorizing approaches to their biological explanation (Tinbergen 1963). 'Tinbergen's four questions' summarize the 'lenses' through which behaviors can be viewed, providing four comprehensive and non-exclusive perspectives applicable to any behavioral phenomenon. These levels of explanation are:

- *Biological mechanism (causation)*: the immediate physiological cause(s) of the observed behavior. Separate from any consideration of the behavior's function or its adaptive significance, one might ask what mechanism brings about its expression [e.g. what sorts of molecules are produced to influence the functions of a host's central nervous system during manipulation by a parasite? (Biron et al. 2005)].
- *Function (survival value)*: what advantage(s) is (are) derived from a particular behavior. For example, an insect might increase its grooming as a response to encountering a parasite infective stage because this reduces the probability of the parasite successfully establishing an infection (Gaugler et al. 1994). In the study of behavioral changes resulting from parasitism, the value of the host behavior may be studied from the host's or the parasite's perspective. Indeed, identifying whether the parasite significantly benefits from the behavior is the crux of differentiating true host manipulation from infection-induced behavioral changes more generally.
- *Evolution (phylogeny)*: the explanation of a behavioral phenomenon from consideration of the organism's evolutionary history. Variation in a behavior expressed by different host species resulting from infection by the same manipulating parasite species may be explained by the degree of phylogenetic relatedness of those hosts (e.g. Malfi et al. 2014). Why individuals of a given species express some particular behavior can also be answered at the level of evolution through addressing how that behavior arose over evolutionary time, and due to what pressures. Selective pressures that favoured the

behavior originally may no longer be relevant, and current survival benefits resulting from the behavior may be relatively new from an evolutionary perspective; therefore, levels of explanation based on function and evolution must be properly distinguished.
- *Ontogeny (development)*: how the observed behavior develops during an individual's lifetime, or is otherwise influenced by the organism's development over the course of its life. For example, one could imagine a scenario wherein an insect's increased exposure to ectoparasites at an earlier life stage increases that individual's propensity to groom at a much later life stage.

Tinbergen's four questions can be divided into two categories depending on whether they deal with 'ultimate' explanations, reasons relating to the purpose of the trait ('why' questions; levels of evolution and function); or whether they address 'proximate' explanations, reasons relating to mechanisms underlying the trait's expression acting within the lifetime of the organism in question ('how' questions; levels of biological mechanism and ontogeny; Klopfer and Hailman 1972). Explanations can alternatively be viewed as either relating to changes occurring over time and to developmental processes (evolution and ontogeny) or, in contrast, relating to more immediate, 'synchronic' perspectives (biological mechanism and function; Bateson and Laland 2013).

Box 18.2 Techniques to study insect behavioral responses to infection

The ability to observe, record, and quantify animal behavior is a considerable challenge in many biological fields. The first challenge arises from the very definition of behavior, which will vary according to the focus of the study (locomotion, courtship, aggression, feeding) and will therefore require distinct methods and techniques for accurate quantification. The second challenge arises from the inherent complexity of behavioral traits. Historically, measuring behavior has required either laborious and time-consuming manual descriptions of behaviors on a limited number of individuals, or has relied on assays that only allow quantification of a simplified aspect of a more complex behavior. While these approaches have advanced the study of behavior, they are prone to inevitable logistic limitations and inherent biases. Here, we briefly describe recent advances in the measurement of insect behavioral responses to infection, with a focus on methods that allow automated, high throughput quantification of individual behaviors. While many of these techniques have been developed using the fruit fly (*D. melanogaster*), they are applicable to many insect species.

Measuring locomotor activity in insects—Trikinetics® Activity Monitor

One of the most popular instruments employed to study activity levels in insects is the Trikinetics® Drosophila Activity Monitor (DAM). The system works by placing insects inside tubes (individually) or vials (in groups) within an activity monitor, and activity is recorded each time active insects break an infrared (IR) beam within the midpoint of each tube (Pfeiffenberger et al. 2010). Its ease of use, relative affordability and the ability to automate the recording of activity on a large number of individuals has made the DAM a popular choice for recording lethargy and somnolence in infected insects such as fruit flies (Shirasu-Hiza et al. 2007; Vale and Jardine 2015), mosquitos (Rund et al. 2016), bees, wasps, and other insects of similar size (Giannoni-Guzmán et al. 2014).

Automated image-based behavioral tracking of locomotor activity in groups of insects

While IR-based tracking systems have a number of advantages, they are limited in their spatial and movement resolution (activity is measured as a binary trait) and their scalability, which requires additional activity monitors. These limitations, allied to the desire of quantifying behaviors in more natural settings, has spurred the development of image and video-based tracking of individual insects while interacting in large groups (Gilestro 2012; Mersch et al. 2013). Some of these approaches are relatively simple. For example, the level of social aggregation in groups of insects (which is relevant for the likelihood of disease spread), is easily measured by using still images of insect groups to measure nearest-neighbour distances (Simon et al. 2012). Actual insect movement, however, tends to occur in three dimensions, which increases the challenge of real-time tracking and quantification (Ardekani et al. 2012). Automated video tracking technology, allied with powerful computational analysis of insect activity data (Egnor and Branson 2016) has revolutionized the field of insect behavior by allowing individual-level behaviors to be linked with higher level ecological patterns (Dell et al. 2014). For example, using continuous tracking of individually-tagged ants within a colony, Mersch and colleagues were able to describe temporal and spatial distribution of all individual ants, and

Box 18.2 *Continued*

identified unique behavioral units within the ant colony that changed over time (Mersch et al. 2013).

Computational analysis of behavior

Automated image-based tracking is complex and extremely data-intensive. Its success as a method to measure behavior will therefore rely heavily on advances in computational analysis (Egnor and Branson 2016). The main challenge is reconstructing the trajectories of individual flies across video frames while accounting for considerable noise both in the measurement and in the assay environment, compounded by the need to maintain the identities of individual insects over time (Ardekani et al. 2012; Reiser 2009). These technical challenges have benefitted greatly from independent advances in the fields of machine learning, which with a solid mathematical grounding, are now being applied to important biological and behavioral processes (Ardekani et al. 2012; Egnor and Branson 2016).

18.2.1 Spatial avoidance

Spatial avoidance occurs when hosts avoid areas where parasites are detected or likely to be. Small-scale spatial avoidance of parasites, as seen for ovipositing insect hosts, is just one type of many avoidance behaviors (Table 18.1). For example, the spreadwing damselfly, *Lestes sponsa*, can lay eggs inserted into aquatic plants, either above or below the water's surface. Below-surface oviposition presumably incurs additional energetic costs to the mother, but offers greater protection of eggs from parasitoids. In the presence of high egg parasitism, females preferentially lay eggs below the water's surface (Harabis et al. 2015).

Some larger-scale movements of insects have been explained in the context of disease avoidance. For example, the migration of the monarch butterfly, *Danaus plexippus*, across North America has been explained partly as a mechanism of avoiding infection (Satterfield et al. 2015; see also Chapter 7). However, this example is somewhat controversial because while migrating populations experience less parasitism (Altizer et al. 2011), it is equally true that parasitized individuals have reduced flight capabilities (Bradley and Altizer 2005) making it unclear whether butterflies migrate to avoid infection, or if infected butterflies migrate less or not at all.

18.2.2 Temporal avoidance

In addition to spatial avoidance, insects also can avoid times where infection risk is highest. Almost all examples of temporal avoidance come from interactions between ants and parasitoid Phorid flies.

Table 18.1 Examples of infection avoidance behaviors (classified according to the subsections of Section 18.2), across a range of insect orders

Insect host order	Parasite	Avoidance behavior	Reference
Coleoptera	Bacteria	Medication	Arce et al. (2012)
Coleoptera	Nematode	Grooming	Gaugler et al. (1994)
Coleoptera	Bacteria and Fungus	Grooming	Lusebrink et al. (2008)
Coleoptera	–	Grooming	Valentine (2007)
Diptera	Parasitoid wasp (Hymenoptera)	Medication	Kacsoh et al. (2013)
Hymenoptera	–	Decreased Social contact	Bigio et al. (2014)
Hymenoptera	Fungus	Niche construction	Chapuisat et al. (2007)
Hymenoptera	Protozoan/Bacteria	Trophic avoidance	Fouks and Lattorff (2011)
Hymenoptera	Fungus	Medication	Konrad et al. (2012)
Hymenoptera	Parasitoid fly	Trophic avoidance	Orr (1992)
Hymenoptera	–	Niche construction	Pie et al. (2004)
Hymenoptera	Fungus	Grooming	Nielsen et al. (2010)
Isoptera	–	Decreased Social contact	Crosland et al. (1997)
Lepidoptera	Multiple (natural populations)	Spatial avoidance	Sadek et al. (2010)
Odonata	Parasitoid wasp	Spatial avoidance	Harabis et al. (2015)

Members of the worker caste of the tropical fire ant, *Solenopsis geminata*, recruit nest mates using pheromone trails to forage *en masse* when large food items are found. Columns of worker ants carrying food can be seen on forest floors, and these high host densities attract parasitoid Phorid flies. In response to the presence of female Phorid flies, worker activity and nest mate recruitment decrease significantly (Feener and Brown 1992). This avoidance behavior also demonstrates the significance of parasite pressure for host ecology, forcing fire ants to balance the cost of reduced foraging efficiency with the benefit of reduced parasitism. A more extreme form of temporal avoidance of Phorid flies is observed in the leaf-cutter ant, *Atta cephalotes*. In the presence of the diurnal Phorid fly, *Neodohrniphora curvinervis*, whole colonies of ants shift their activity from daytime to night-time (Orr 1992). This response to parasitism is co-ordinated by many individuals over a short time period, making the interaction between ants and Phorid flies of fundamental interest to our understanding of adaptation and behavioral plasticity.

18.2.3 Trophic avoidance

Several activities that are essential to survival also increase the risk of acquiring infection. Foraging and consuming food is vital to host health, but offer parasites an ideal route with which to access the internal environment of the host. Many parasites infect the insect digestive system (see also Chapter 21). The cost of this infection is thought to have driven the evolution of trophic avoidance, where individuals avoid eating infectious food items (Alma et al. 2010; Fouks and Lattoroff 2011). Even insects typically thought of as having poor sensory systems, such as the larvae of holometabolous insects, can exhibit trophic avoidance to an array range of parasites. For example, larvae of the grapevine moth, *Lobesia botrana*, avoid eating fungus-infected grapes (Tasin et al. 2012), while larvae of the gypsy moth, Lymantria dispar, avoid leaves where viruses are detected (Parker et al. 2010).

18.2.4 Altered mate preference

Courtship and mating are activities during which infections can be transmitted between individuals. As a result, many hosts avoid mating with infected conspecifics. This mate avoidance is commonly seen in vertebrates, but is rarely seen in insects (Abbot and Dill 2001; Rosengaus et al. 2011), despite the fact that it has now been tested for across several insect host systems (Arbuthnott et al. 2016). The rarity of altered mate preference in insects in relation to parasitism is puzzling as traits that are linked to parasitism have been shown to be targets of mate choice, e.g. a number of sexually selected traits have demonstrable ties to immunocompetence (Siva-Jothy 1999; Tregenza et al. 2006). Therefore, the overall rarity of altered mate preferences must have another explanation, such as parasites evading host detection (Lambardi et al. 2007), parasites generally manipulating host behavior (but see Section 18.3) or the cost of abstinence outweighing the cost of infectious mating.

Microbiota generally have a range of effects on host–parasite dynamics and have been shown to influence mate choice (Arbuthnott et al. 2016; Damodaram et al. 2016). That insects can detect these microorganisms and incorporate this information into mate choice, makes general parasite evasion of host detection less likely (de Roode and Lefèvre 2012). Particularly for microorganisms such as *Wolbachia* causing host cytoplasmic incompatability, there is expected to be intense selection on detection as uninfected female hosts mating with infected males have zero fitness. The cost-of-abstinence argument, in comparison, makes sense in light of insects being relatively short-lived organisms with a premium on mating and given that this form of infection avoidance is so commonly seen in longer-lived vertebrates.

18.2.5 Decreased social contact

Insects also exhibit a range of non-sexual interactions (see Chapter 15). These interactions can be exploited by parasites to infect hosts. Literature detailing the influence of parasites on these social interactions focuses on eusocial insects, due to the significance these interactions have on colony fitness (Cremer et al. 2007). Workers increase their fitness through kin selection; protecting and providing for reproductive nest mates (see Chapter 15). Selection favours workers preventing transmission to the rest

of the colony even if that individual increases its own short-term infection risk (Cremer et al. 2007). The termite, *Reticulitermes fukienensis*, for example, undertakes a range of behaviors in order to limit contact with infected workers or workers that have died from infection. One marked example is the burying of nest mates that died from fungal infection (Crosland et al. 1997). In so doing, workers reduce infection risk to other nest mates.

Social organization of eusocial insect colonies also serves to reduce infection risk. For example, worker castes of the carpenter ant, *Camponotus fellah*, take on a range of tasks that have variable infection risk (Mersch et al. 2013). Although the effect of infection risk was not directly tested, worker castes central to colony reproduction, i.e. brood nurses, seldom interacted with castes that performed jobs with a high risk of infection (Mersch et al. 2013). This aids brood nurses in safely rearing the colony's next generation.

18.2.6 Niche construction and maintenance

As many parasites can persist in the environment without hosts, insects can avoid infection by making and maintaining their environment to be less hospitable to infective stages of parasites. Nest-making insects frequently dedicate time or, in the case of eusocial insects, castes to the removal of parasites or infectious material from the nest (Cremer et al. 2007; Neoh et al. 2012; Bigio et al. 2014). Other factors that reduce parasitism risk include antimicrobial secretions (Turillazzi et al. 2006; Chapuisat et al. 2007) and nest architecture (Pie et al. 2004).

The burying beetle, *Nicrophorus vespilloides*, raises its offspring on small vertebrate carcasses and employs antimicrobial secretions. The carcasses attract microparasites that can affect offspring survival and development adversely. This cost has driven the evolution of antimicrobials secretion by parents, while preparing their offspring's food source (Arce et al. 2012).

Nest architecture is diverse in many social insects. Nests comprised of multiple chambers limit interactions between castes to focal individuals or specialized castes (Mersch et al. 2013). Additionally, chambers can be dedicated to specific functions, for example, the nests of the social cricket, *Anurogryllus muticus*, have dedicated latrine chambers (Curtis 2014).

18.2.7 Grooming

Grooming is a mechanism of infection avoidance that shares activities such as those described above, but it primarily concerns maintenance of the insect's cuticle. Grooming reduces infection risk by removing parasites from, or preventing their establishment on, the cuticle (Gaugler et al. 1994; Turillazzi et al. 2006). Individuals of many insect species groom themselves (autogrooming) and one another (allogrooming; Valentine 2007). Grooming is even observed between species, as is the case in the aphid-farming ant, *Formica podzolica*, which grooms its aphid-livestock (Nielsen et al. 2010). Insects have a number of adaptations that increase grooming efficiency including specialized bristles (Zhukovskaya et al. 2013) and cuticular secretions (Lusebrink et al. 2008). For example, the rove beetle, *Stenus comma*, secretes the alkaloid, stenusine from specialized abdominal glands to reduce the growth of fungi, Gram-positive and Gram-negative bacteria on the cuticle (Lusebrink et al. 2008).

Interestingly, secretions are used alternatively by insect parasites frequently to evade hosts. The cuckoo bee, *Bombus bohemicus*, infiltrates host hives by mimicking that colony's cuticular secretions, where it goes on to monopolize resources for reproduction and suppress host reproduction (Kreuter et al. 2012). In comparison, the social parasite, *Acromyrmex insinuator*, infiltrates host nests by appearing 'chemically insignificant', not secreting cuticular hydrocarbons or any other pheromones (Lambardi et al. 2007). It appears that hosts do not use cuticular secretions to evade parasites for as yet unknown reasons.

18.2.8 Medication

Just as insects produce compounds that reduce infection, they can also apply or consume compounds found in their environment for a similar purpose. When done prior to infection, this is known as **prophylactic** medication and, in insects, typically occurs between siblings or parents and offspring. If insects are already infected, using compounds that help clear infection is known as therapeutic medication (see Section 18.3.4).

Parent–offspring prophylaxis occurs during *D. melanogaster* oviposition in the presence of parasitoid wasps (Kacsoh et al. 2013). In the absence of

wasps, mothers avoid laying eggs in sites with a high ethanol content due to the detrimental effect of alcohol on offspring development. However, ethanol confers increased larval avoidance of parasitoid infection. As a result, if mothers see parasitoid wasps, they preferentially oviposit in high-ethanol sites (Kacsoh et al. 2013).

Sibling–sibling prophylactic medication occurs in the ant, Lasius neglectus, where individuals infected with the fungal parasite, *Metarhizium anisopliae*, transfer small quantities of the fungus to susceptible nest mates. The transfer of fungus acts as an elicitor for **immune priming** rarely killing workers, but regularly decreasing susceptibility to subsequent fungal infection by upregulating fungal immune genes (Konrad et al. 2012). Socially inoculated colonies are far less susceptible to outbreaks of *M. anisopliae* infection.

18.2.9 Integrated studies of infection avoidance

Studies of infection avoidance typically focus on the broader ecological consequences of the behavior due to its consequences for host–parasite interactions. Studying the mechanisms of these avoidance behaviors enables the discovery of their physiological mediators and, by extension, the constraints on their evolution. Recent advances in neurobiology and endocrinology have significantly increased the detail with which we can study such mechanisms (Kohmura et al. 2015) (see Box 18.3).

Box 18.3 The mechanistic basis of behavioral changes in infected insects: the neuroendocrine-immune axis

Behavioral changes in response to infection appear to be taxonomically conserved within animals and especially within insects. However, two major questions remain (Lopes 2017):

- What are the mechanisms by which behavior and immunity are linked (how)?
- Why would behavior and immunity be linked at all (why has this link evolved)?

Both proximate and ultimate questions can be addressed by considering that infection is essentially an extreme form of physiological stress, and that the immune and central nervous systems are interconnected by the endocrine system, forming the major neuroendocrine-immune axis which regulates both behavior and immunity in response to pathogens (Adamo 2014).

The physiological response of organisms to stress, such as fight or flight behaviors, is mainly coordinated by the release of neuroendocrine factors, which appear to be largely conserved across vertebrates and invertebrates, and similar stress hormones mediate both endocrine and immune systems of many arthropods, including crustaceans, molluscs, and insects (Adamo 2006). In insects, the major neurohormone released during stress and fight-or-flight behaviors is octopamine (OA). Similar to the effect of its mammalian homologue norepinephrine (NO), the release of OA during stress in invertebrates results in the release of energetic resources, and increases responsiveness to external threats. Approximately 30 minutes after the release of OA from the dorsal unpaired medial (DUM) cells (essentially the equivalent to insect neurons), a peptide called adipokinetic hormone (AKH) is released by the corpora cardiacum, an insect endocrine organ. AKH plays an important role in mobilizing lipids and releasing energy for costly activities, such as flight (Adamo 2006; Adamo 2017).

The role of OA during infection in insects has received considerable attention (Adamo 2006, 2014). Haemocytes, invertebrate immune cells, have OA cell surface receptors (Huang et al. 2012), which allows direct neuroendocrine modulation of immune function. Overall, the effects of OA on insect immunity appear to be immune enhancing, although some immune suppressive effects also have been reported. For example, injection of high physiological doses of OA tends to increase haemocytes growth and phagocytocis, but injections low physiological doses OA make the cricket (*Gryllus texensis*) more susceptible to bacterial infection. AKH has also been directly linked to changes in immune function, but again its effects appear to be highly context dependent. For example, in the African migratory locust, *Locusta migratoria*, AKH increased phenoloxidase activity after an immune challenge enhancing immune function but also reduced antimicrobial activity, increased bacterial growth in the hemolymph and increased susceptibility to the fungus *Metarhizium anisopliae* (Goldsworthy et al. 2002).

The OA-AKH cascade therefore provides a possible evolutionary explanation for the link between behavior and immunity—if immune responses are energetically costly, the neuroendocrine mechanisms that have evolved to allocate resources to other costly behaviors (like fight-or-flight) may also have been co-opted to do the same during an immune challenge (Adamo 2017).

Drosophila melanogaster is a model organism particularly well suited to this research due to the suite of molecular tools available in the study of its neurobiology, genetics and behavior. As previously mentioned, prophylactic medication is elicited in *D. melanogaster* by the visual cues of the parasitoid wasp, *Leptopilina heterotoma* (Kacsoh et al. 2013). Using genetically modified flies, with fluorescently-labelled neural circuits, researchers detailed the specific neural pathways and neuropeptide mediating this oviposition choice behavior (Kacsoh et al. 2013). This study went on to also examine the evolution of this avoidance, measuring it in six other *Drosophila* spp., revealing multiple independent evolutions within the genus. *D. melanogaster* has also been used to detail dedicated olfactory circuits for harmful microbes (Stensmyr et al. 2012) alongside the signatures of such sensory systems in the genome (McBride and Arguello 2007).

18.3 Behavioral changes in infected insects: sickness behaviors as host adaptations

Until now the variation in insect behavior in response cues of infection from infectious conspecifics or environments that may increase the risk of infection has been described. If avoiding these sources of infection is unsuccessful and individuals become infected, a suite of behavioral changes may also be observed in infected animals. The most common behavioral changes in infected animals are manifested in alterations in activity levels (increased lethargy) and sleep patterns (increased somnolence)—decreased foraging behavior and reduced food intake (anorexia), and a lower libido and reduced investment in sexual reproduction (Hart 1988, 2011; Moore 2013).

In some cases, the simplest explanation for these behaviors is that they arise as a direct consequence of the pathology either due the direct damage caused by pathogen growth, or because of the energetic expenditure arising from mounting an effective immune response (Moore 2013). In other cases, behavioral changes in infected hosts are the result of intricate pathogen strategies to manipulate the way an infected host behaves, which enhance a pathogen's evolutionary fitness by increasing the chances of successful transmission to new hosts (Poulin 2010). We will describe these **host manipulations** in greater detail in the following section. A third potential explanation for changes in host behaviors during infection, however, is that they reflect adaptive sickness behaviors that allow animals to conserve energetic resources during infection.

This adaptive hypothesis for sickness behaviors was originally proposed by Benjamin Hart (Hart 1988), and posits that energy not expended by infected animals in finding food, or finding a mate could, instead, be reallocated to immunity, thereby increasing the chances of clearing the infection and recovering health. One of the arguments put forward for the adaptive nature of sickness behaviors is that lethargy, somnolence, and reduced reproduction appear to be evolutionarily conserved responses to infection across a wide range of taxa (Moore 2013; Sullivan et al. 2016).

In addition to quantifying the possible fitness benefit of sickness behaviors, it is also important to consider potential indirect fitness costs. For example, reducing activity during infection may conserve energy that is allocated to fighting infection, but also means that animals are forced to reduce other fitness-enhancing activities such as foraging, courtship and mating, parental care, and territorial defence, and may even leave individuals more susceptible to predation (Adelman and Martin 2009; Lopes 2014; Vale and Jardine 2016). As discussed next, insects have become central to addressing both the benefits and potential costs of sickness behaviors (de Roode and Lefèvre 2012; Sullivan et al. 2016).

18.3.1 Infection-induced lethargy

One of the most commonly observed behaviors in sick animals is a decrease in activity following infection (lethargy). For example, fruit flies, *Drosophila melanogaster*, show reduced daily locomotor activity when infected with Drosophila C Virus (DCV; Vale and Jardine 2015). While lethargy may simply be a consequence of being sick, reduced activity may also bring benefits if energetic resources are instead allocated to immune defence. For example, honey bees (*Apis mellifera*) challenged with **lipopolysaccharide (LPS)** mount a strong immune response and subsequently exhibit a reduction in locomotor activity, suggesting that reduced activity results from

a reallocation of resources to immunity (Kazlauskas et al. 2016).

18.3.2 Decreased social contact and isolation

Active individuals are also more likely to be more gregarious and to partake in activities with other conspecifics, such as courtship, mating, and fighting (Lopes 2014). Reduced activity will therefore lead to decreased social interactions, thereby decreasing the likelihood of encountering sources of infection. We may therefore expect that risk of disease (and the resulting fitness costs of acquiring infection) should select for behaviors that lead to less gregarious individuals. The effects of infection on social aggregation have been found to be especially clear in eusocial insect systems that are strongly influenced by kin selection (Cremer et al. 2007). In the same honey bee example described previously, bees pricked with bacterial-derived LPS spent more time alone, standing still, or self-grooming, and showed reduced social contact with other bees (Kazlauskas et al. 2016). Another popular example of changes in social aggregation following infection occurs in worker ants, *Temnothorax unifasciatus*, which when infected with a pathogenic fungus change their social behavior by leaving the nest permanently (Heinze and Walter 2010). While this could initially appear to be a form of host manipulation by the fungus, by leaving the nest long before death, this behavior is likely to reduce the spread of fungus, possibly curtailing pathogen transmission.

18.3.3 Infection-induced anorexia

The degree to which infection is detrimental to hosts is often affected by their physiological status (Adamo 2009), which in turn is greatly impacted by the quantity and quality of host diet (Ponton et al. 2013; Singer et al. 2014). Behavioural changes affecting the quantity of food intake are particular common and animals commonly exhibit a loss of appetite (anorexia) following infection (Hart 2011; see also Chapter 8). Given the heavy energetic burden of immune responses, this behavioral change effectively reducing caloric intake may seem counterintuitive. However, reduced food intake has been shown to increase recovery from infection, and is a conserved behavior across many vertebrates and invertebrates (Murray and Murray 1979; Ayres and Schneider 2009; de Roode and Lefèvre 2012).

Work in insect host–pathogen systems is starting to address the complex link between nutritional intake and invertebrate immunity. For example, fruit flies, *D. melanogaster*, infected with either *Salmonella typhimurium* or *Listeria monocytogenes* have been shown to become anorexic (Ayres and Schneider 2009). However, this change in feeding behavior appears to have different effects on fly immunity according to the type of infection. When infected with *L. monocytognes*, anorexia reduced the ability of the fly to clear infection, while anorexia during *S. typhimurium* infection increased **infection tolerance** because flies did not improve their ability to clear infection, but still survived longer. These contrasting effects of infection-induced anorexia appear to occur because restriction of nutrition affect cellular and humoral immune responses differently, suggesting that in the wild we should expect this sickness behavior to vary with the prevalence of specific pathogen types (Ayres and Schneider 2009).

In addition to the quantity of food, infected animals may also alter the type and quality of food they choose to ingest (see also Chapter 8). For example, diet-induced changes in susceptibility may be determined by the precise ratio of specific macronutrients. The survival of the Egyptian leafworm, *Spodoptera littoralis*, when infected with a nucleopolyhedrovirus (NPV) depends strongly on the protein:carbohydrate ratio in its diet, and insects fed on a high-protein, low-carbohydrate diet showed the highest survival following NPV challenge (Lee et al. 2008). Furthermore, when insects were given a free choice of diets containing different ratios of protein:carbohydrate, larvae that *selected* a diet containing higher protein content lived longer than those choosing alternative diets. Behavioural changes that affect what and how much an insect chooses to eat when infected can therefore have important effects on how sick it gets and how likely it is to recover (Singer et al. 2014).

18.3.4. Dietary self-medication

Dietary choice following infection suggests that infected insects are capable of dietary self-medication

(see Chapter 8), by choosing food sources that enhance their chances of survival (de Roode et al. 2013). Self-medication can occur through nutritional effects that enhance the host's ability to fight infection, or by the direct anti-parasitic properties of the ingested compounds. Insects have emerged as powerful systems to conduct manipulative experimental tests of the occurrence and potential benefits of dietary self-medication (de Roode and Lefèvre 2012).

For instance, when *Grammia incorrupta* caterpillars are infected by a parasitoid, they show an increased preference for alkaloid toxins found in larval food plants, and by choosing to eat these toxic plants when infected, are able to increase survival. Using self-medication to ensure the survival of offspring also has been shown in Monarch butterflies. Several studies have shown that milkweeds—the plant species that Monarch butterflies use as their larval food plants—increase the survival of butterflies infected with the protozoan *Ophryocystis elektroscirrha*. While infected larvae did not preferentially consume medicinal milkweed, preferential oviposition on medicinal milkweeds by infected females resulted in reduced parasite growth in the offspring (Lefèvre et al. 2010).

18.3.5 Behavioural thermoregulation

Mammals and other endotherms can regulate their own body temperature and fever is a commonly observed response to infection. Ectotherms, such as insects and other invertebrates, can elevate temperature when infected by seeking out warm locations that allow their body temperature to rise to levels that may be detrimental to pathogens, called **behavioral fever** (Thomas and Blanford 2003; de Roode and Lefèvre 2012). Behavioural fever is a widespread behavioral response to infection, and has been especially well documented in insects (Stahlschmidt and Adamo 2013). For example, heat-seeking behavior has been documented in house flies, grasshoppers, and in the desert locust infected with entomopathogenic fungi (Blanford et al. 1998; Kalsbeek et al. 2001; Elliot et al. 2002), and in honeybees, who communally raise the temperature of their hive in response to an infection with the heat-sensitive pathogen that causes chalkbrood (Starks et al. 2000).

In addition to increasing body temperature, behavioral thermoregulation can also be used to lower temperature, which can delay parasite growth. Examples of cold-seeking behavior have been reported in fruit flies, *D. melanogaster*, infected with the fungal pathogen, *Metarhizium robertsii*; cold-seeking comes at an initial cost of lower reproduction, but slowing fungal growth ultimately results in higher lifetime reproduction for flies (Hunt et al. 2016). Similar growth-retarding effects of cold-seeking behavior occur in acanthocephalan-infected cockroaches (Moore and Freehling 2002), and in bumblebees infected with thick-headed flies (Conopidae). Instead of spending the night in the warmth of the hive, observational and experimental work has shown that infected bumblebees achieve increased survival by preferentially spending time in cold areas, which reduces parasite growth rate (Müller and Schmid-Hempel 1993).

18.4 Behavioural changes in infected insects: host manipulation as a parasite adaptation

Parasite-induced changes in host behavior, often interpreted as parasite adaptations to aid in their own transmission and/or survival, are widespread amongst parasite–host associations, but not universal (Heil 2016). Some of the most celebrated examples of host manipulation come from associations where insects are the host species, or both the host species and parasitoid. These widespread and recurring phenomena provide considerable scope to examine the significance of host manipulation with respect to the Tinbergen's four levels of behavioral explanation (Box 18.1b).

Poulin (1995) initially outlined four criteria indicative of adaptive parasite-induced behavioral changes in hosts. They include the following:

- the induced behavior should be complex;
- it should align with clear a priori expectations for its potential benefit to the parasite, i.e. demonstrate obvious purpose;
- it may arise independently in multiple host and parasite lineages by convergence;
- the induced behavior should increase the fitness of the parasite.

However, it is really only the last criterion, that there be a fitness benefit to the parasite, which decisively designates a behavioral change as adaptive manipulation (Poulin 2010).

In such studies, therefore, we tacitly focus on how the parasite's genes and gene products might enhance its fitness through the extended behavioral phenotype of its host species. By extension, we are examining the degree to which parasites are locally and temporally adapted to their insect hosts. It is important to remember that for any observational or experimental study of behavioral responses of host individuals to parasite or pathogen infection, the intensity of the host's response could be due to parasite-related factors, host-related factors and environmental factors acting alone or in combination. The parasite- and host-related factors can be further subdivided into genetic versus non-genetic characteristics of the parasite or host (Thomas et al. 2012). Disentangling the various causes of the altered behavior can help researchers interpret whether the altered behavior is mainly a host adaptation to curb impacts of infection (see previously, host in control), a true parasite manipulation of host behavior benefitting the parasite, or a by-product of infection (as mentioned, neither host nor parasite is in control, and the behavioral change might be costly or beneficial to either or both).

For students of insect behavior, the altered behaviors of parasitized insect hosts that fall under the rubric of host manipulation are sometimes subtle, sometimes strange and curious, as the following examples will illustrate. Each of the examples with insect hosts was chosen to further illustrate a key concept(s) concerning the study host manipulation by parasites.

18.4.1 Manipulation of concealment behaviors of parasitized hosts

The life cycle of the liver fluke, *Dicrocoelium dendriticum*, makes for an enjoyable read. The case for adaptive manipulation of ants by larvae of this worm was made over 55 years ago [by Hohorst and Graefe (1961), cited in Hölldobler 2012]. Importantly, this liver fluke is a cosmopolitan parasite of grazing mammals: adults of this trematode reside in the liver of sheep, cattle, pigs, goats, and cervids (Goater et al. 2014). The eggs are passed with the faeces (via the bile duct) and ingested by terrestrial snails, which later egest cercaria (larval flatworms) in a mucous mass. Ants of various species eat the mucous and ingest the larval worms, some of which encyst in the ant's abdomen and at least one of that migrates to the suboesophagial ganglion of the ant, but does not encyst and therefore is not transmissible (Goater et al. 2014). This brain worm 'turns on' a stereotypical behavior of the infected ant by its climbing vegetation and clamping its mandibles down on a leaf, flower, or blade of grass. These solitary anchored ants are susceptible to incidental ingestion by grazing mammals, wherein the parasites later excyst in the small intestine and travel to the bile duct to develop and reproduce.

This iconic example of host manipulation illustrates two important points. The first is that apparent maladaptive host behavior can make sense. Such height- or open-seeking (non-concealment) behavior has been described for other diverse associations, e.g. ants parasitized by fungi (*Ophioccordyceps* spp.) and flour beetles parasitized by nematodes. However, researchers still have to test whether the altered behavior actually leads to increased transmission success. For example, Schutgens' et al. (2015) excellent work uncovered behavioral changes in the flour beetle, *Tribolium confusum*, infected with the spirurid nematode, *Protospirura muricola*. Infected beetles took longer to conceal themselves in experimental trials and spent less time concealed. Infected beetles (particularly those with >1 cyst) also were more likely to be in an illuminated part of the trial arena. These differences were observed for hosts with older and not younger cysts. All these findings support the hypothesis that ontogenetic changes in infection-adjusted behavior are adaptive by increasing successful transmission to the definitive host. However, these experiments were not conclusive in this respect—host behavioral changes might simply be related to infection pathology, and no predation experiments with actual definitive hosts were performed, wherein the success of parasite establishment following infection was assessed.

The second point is that there are an astounding number of natural experiments of 'evolution in action', even with just this one parasitic worm species. Given its worldwide distribution, there are

probably many different species of snails and ants that act as intermediate hosts. Add to this the fact that wild and domestic grazing mammals also vary from place to place. There is probably a wide array of efficacies in the ant stereotypical behavior depending on how well adapted larval trematodes are to their insect host and the extent to which the infected insects secure a place and a time where/when it is likely to be ingested by suitable definitive host.

18.4.2 Increased defensiveness of moribund hosts protecting parasitoids

It is a hallmark of many systems involving host manipulation by parasites that the manipulation results in the host death, either through ingestion of infected prey by final hosts as described above for the liver fluke, or by other means such as drowning in the iconic case of cricket infected with horsehair (nematomorph) worms (Thomas et al. 2012). A unique form of **behavioral manipulation** is described by Harvey et al. (2011) who show that host armyworm that are moribund (near death) following emergence of parasitoid wasps protect the wasps against hyperparasitism by repelling other parasitoid wasps (see Figure 18.1 for a related example). The initial parasitoids presumably benefit from a moderate level of virulence leaving moribund, but not dead caterpillars to protect them. Another example of this phenomenon concerns a solitary parasitoid wasp, *Dinocampus coccinellae*, parasitizing a ladybird beetle, *Coleomegila maculata*. The larval parasitoid emerges as a late instar from the ladybird beetle and spins a cocoon for pupation between the ladybird's legs. The ladybird beetle is moribund, but defends the cocoon against predatory lacewings, *Chrysoperla carnea*, which are common (Thomas et al. 2011). More recently, there is genetic and microscopic evidence that time-related changes in behavior of the ladybird beetle is due to replication/clearance of a virus acquired from the parasitoid (Dheilly et al. 2015).

18.4.3 Manipulation of host sexual or social behavior

Work by Burand et al. (2004, 2005) on the corn earworm moth infected by Hz-2V virus shows morphological and physiological changes in females, coupled with behavioral changes of mate searching males. Infected females continue to attract males following first contact, i.e., they do not become refractory. What is interesting is that these females are agonadal, but still attract males, probably due to the heightened production of pheromones. The virus-infected females are highly attractive to mate searching males, which pick up the highly contagious plugs and transfer them to uninfected female. The virus thus induces a change in the behavior of the males towards infected females—a form of indirect manipulation.

In his provocative review, Heil (2016) raised the issue that there are few examples of host manipulation by sexually transmitted pathogens and parasites (STDs). This might be because the costs of abstinence (see Section 18.2.4) ensure that host sexual activity is high enough to ensure transmission or it might be that the STDs have evolved not to invoke host sickness or asocial behaviors.

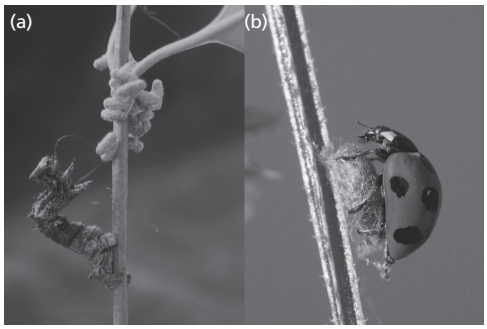


Figure 18.1 Two cases of behavioural manipulation by parasitoids in which an insect host defends an emerged parasitoid during the latter's pupation, i.e. examples of hosts expressing modified behaviour after infection has ceased. (a) A *Thyrinteina leucocerae* caterpillar stands guard below a cluster of braconid (*Glyptapanteles* spp.) pupae, which previously emerged from it following an approximately 2-week period of infection. The affected caterpillar displays defensive behaviours when disturbed (vigorous thrashing of its head) for the 6 or 7 days before it dies following egression of the parasitoid larvae, and otherwise remains practically motionless (Grosman et al. 2008; adapted from a photo by José Lino-Neto, CC BY 2.5). (b) A single braconid (*Dinocampus coccinelae*) pupa is protected underneath the coccinellid (ladybeetle) host from which it emerged. While guarding the parasitoid, the ladybeetle displays tremors, particularly when disturbed, but otherwise remains paralysed. Approximately 25% of these beetles survive the entire parasitoid pupation period (~7 days) and then regain expression of normal behaviour (Thomas et al. 2011; adapted from a photo by conifer on Flickr, CC BY 2.0).

18.4.4 Manipulation of behavior of insect vectors

There are several studies in the primary literature showing effects of microparasites on the feeding behavior of their insect vectors of human diseases, including sand flies and Leishmanias, Tsetse flies and African sleeping sickness, mosquito vectors, and malaria (reviewed by Hurd 2003). One of the points made is that the parasite may influence the vector in ways that increases vectoring ability (parasite's interest) even if it poses a risk to the insect vector's survivorship. For example, the study by Botto-Mahan et al. (2006) showed kissing bugs infected with Chagas disease demonstrated more rapid host detection, increased biting rate and shortened time to defecation (defecation facilitates transmission of the protozoan; see Chapter 21). Chagas disease infects an estimated 10–12 million people in South and Central America (Goater et al. 2014). Some understanding of the subtleties of apparent manipulation of vector risky behavior can help inform epidemiological models. Other studies on potential vector manipulated behaviors with an applied thrust include studies such as aphids and cucumber plants harbouring cucumber mosaic virus. Here, researchers are interested in uninfected aphids being attracted to virus laden plants and then recording the later behavior of the infected aphids in terms of whether they are attracted to already infected plants versus uninfected plants, as a means to promote viral transmission (Carmo-Sousa et al. 2014). The reader is referred to McMenemy et al. (2012) for similar results with raspberry viruses.

18.4.5 Manipulation in an ecological context

Lafferty and Kuris (2012) outline some examples of where host manipulation by parasites has far-reaching effects on community organization. An insect example of this phenomenon concerns the horsehair worm manipulation of orthopteran insects. Manipulated crickets and grasshoppers drown and become food for trout. The manipulated insects can account for up to 60 per cent of the trout's energetic needs, according to work by Sato et al. (2012, and references therein). These easy prey result in less predation pressure on benthic invertebrates, which means streams frequented by parasitized insects have more diverse and abundant communities of benthic invertebrates (Sato et al. 2012). Although the host drowning is beneficial for the horsehair worm, ingestion by trout would not be for the free-living adult nematomorph worms. Perhaps this is why the behavioral manipulation is timed to occur at night and the worms exit the drowning hosts, rather quickly less they also become prey for foraging fish.

18.4.6 Manipulation in an evolutionary context

Ever since Poulin's (1995) seminal paper, researchers have emphasized that the evolution of host manipulation by parasites needs to be understood in a phylogenetic context. Here, the manipulative behavior of a parasite could have been inherited from an ancestor, and might only work partially in the current context and provide examples of 'evolution in action'. Acanthocephalans, for example, are comprised entirely of manipulative species: that is, they have been shown to alter the behavior of their intermediate hosts to make predation by definitive hosts more likely. However, whether predation by appropriate definitive hosts is increased as a result of the manipulation is a tall order often missing from studies, as mentioned already. Another related question is the extent to which different host species differ with respect to behavioral modification following parasitism. Malfi et al. (2014) reported that bumblebee self-burying behavior is observed across several host species infected with the same parasitoid canopid fly (burying enables fly pupation), although there is variability in this response—a higher likelihood of self-burying in response to infection was observed in two closely related species (same subgenus), while lower probability of self-burying was observed in a less closely related bumblebee species.

18.5 Concluding remarks: future directions in the study of insect behavior in relation to parasites

It is clear that insects have provided a wealth of examples of behavioral responses to (risk of) infection that can be classified as avoidance behavior pre-infection, adaptive host sickness behavior following infection, and adaptive parasite manipulation following infection. Each of these areas of

investigation is ripe for future research that focuses on integration of mechanism with investigations of proposed function. Only then can serious claims that responses are not a by-product of infection be made. We suspect that there are many cases in nature of 'evolution in action' where partial solutions to the problems of risks or costs of infection or parasite transmission/survival will be observed and longitudinal studies largely absent from this review are welcomed. It is also important to ask in this context why a predicted behavior is not present when it is expected to be, such as is the case with mate avoidance in insects in relation to parasitism risk.

What follows is a brief overview of some of the main questions still outstanding. In addition to the need for integrated studies such as those cited at the end of the parasite avoidance section, researchers can explore the extent to which behavioral responses are plastic or context dependent, e.g. how an infection avoidance behavior might vary, depending on virulence or infection risk. A plastic infection avoidance response was experimentally demonstrated in the pollination behavior of the bumblebee, *Bombus terrestris* (Fouks and Lattorff 2011). This was done using two parasites of differing virulence, the more virulent bumblebee specialist, *Crithidia bombi* and the less virulent generalist parasite, *Escherichia coli*. Congruous with a plastic infection avoidance response, *B. terrestris* avoided flowers contaminated with *C. bombi*, significantly more than *E. coli* (Fouks and Lattorff 2011). Considering how the plasticity of other infection avoidance behaviors affects their evolution and influence on host–parasite dynamics goes hand-in-hand with furthering our understanding of their mechanistic basis. Plasticity's significance to evolutionary biology has dramatically increased in recent years, with some arguing it warrants a drastic shift in how we conceptualize evolutionary processes (Laland et al. 2014). Not only are these dynamics probably central to infection-avoidance behaviors, but they are also an ideal context with which to test their significance.

Secondly, it is extremely difficult to measure sickness behaviors in the wild on individuals who have contracted infections naturally. However, the aforementioned longitudinal studies are needed to assess variation in prevalence and intensity of parasitism in order to design realistic experiments, but also to assess the costs of retaining particular 'sickness' behavioral responses if the threat of parasitism is at best intermittent. A combination of field assessments and common garden experiments might prove fruitful for understanding the evolutionary dynamics of apparent sickness behaviors. Such studies will also have to consider that the indirect costs of sickness behaviors (reduced time spent foraging or searching for mates) might vary from place to place and time to time.

Thirdly, researchers might wish to explore or at least discuss the evolutionary trajectories that have led to particular responses. For example, trematodes alter the behavior of their hosts by becoming encysted in the host brains. This location might have been favoured early on by providing the parasite with a host immunity barrier. Another question of priority event concerns the hairworm. Adults mate in water and larvae manipulate their insect hosts toward suicidal drowning behavior. Thomas et al. (2012) question whether the behavioral manipulation preceded mating in water or whether mating in water provided the context by which behavioral manipulation was subsequently selected for. It is our hope that these and other issues in the study of insect behavior in relation to parasitism will continue to generate much interest.

References

Abbot, P., and Dill, L.M. (2001). Sexually transmitted parasites and sexual selection in the milkweed leaf beetle, *Labidomera clivicollis*. *Oikos*, **92**, 91.

Adamo, S.A. (2006). Comparative psychoneuroimmunology: evidence from the insects. *Behavioral and Cognitive Neuroscience Reviews*, **5**, 128.

Adamo, S.A. (2009). The impact of physiological state on immune function in insects. In: S. Reynolds and J. Rolff (Eds), *Insect Infection and Immunity*, pp. 173–86. Oxford University Press, Oxford.

Adamo, S.A. (2014). The effects of stress hormones on immune function may be vital for the adaptive reconfiguration of the immune system during fight-or-flight behavior. *Integrative and Comparative Biology*, **54**, 419.

Adamo, S.A. (2017). The stress response and immune system share, borrow, and reconfigure their physiological network elements: evidence from the insects. *Hormones and Behavior*, **88**, 25.

Adelman, J.S. and Martin, L.B. (2009). Vertebrate sickness behaviors: adaptive and integrated neuroendocrine

immune responses. *Integrative and Comparative Biology*, **49**, 202.

Alma, C.R., Gillespie, D.R., Roitberg, B.D., and Goettel, M.S. (2010). Threat of infection and threat-avoidance behavior in the predator *Dicyphus hesperus* feeding on whitefly nymphs infected with an entomopathogen. *Journal of Insect Behavior*, **23**, 90.

Altizer, S., Bartel, R., and Han, B.A. (2011). Animal migration and infectious disease risk. *Science*, **331**, 296.

Arbuthnott, D., Levin, T.C., and Promislow, D.E.L. (2016). The impacts of *Wolbachia* and the microbiome on mate choice in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **29**(2), 461–8.

Arce, A.N., Johnston, P.R., Smiseth, P.T., and Rozen, D.E. (2012). Mechanisms and fitness effects of antibacterial defences in a carrion beetle. *Journal of Evolutionary Biology*, **25**, 930.

Ardekani, R., Biyani, A., Dalton, J.E., et al. (2012). Three-dimensional tracking and behaviour monitoring of multiple fruit flies. *Journal of the Royal Society Interface*, 20120547.

Ayres, J.S., and Schneider, D.S. (2009). The role of anorexia in resistance and tolerance to infections in Drosophila. *PLoS Biology* **7**, p.e1000150.

Bateson, P., and Laland, K.N. (2013). Tinbergen's four questions: an appreciation and an update. *Trends in Ecology and Evolution*, **28**, 712.

Bigio, G., Al Toufailia, H., and Ratnieks, F.L.W. (2014). Honey bee hygienic behaviour does not incur a cost via removal of healthy brood. *Journal of Evolutionary Biology*, **27**, 226.

Biron, D. G., Marché, L., Ponton, F., et al. (2005). Behavioural manipulation in a grasshopper harbouring hairworm: a proteomics approach. *Proceedings of the Royal Society B*, **272**, 2117.

Blanford, S., Thomas, M.B., and Langewald, J. (1998). Behavioural fever in the Senegalese grasshopper, *Oedaleus senegalensis*, and its implications for biological control using pathogens. *Ecological Entomology*, **23**, 9.

Botto-Mahan, C., Cattan, P.E., and Medel, R. (2006). Chagas disease parasite induces behavioural changes in the kissing bug *Mepraia spinolai*. *Acta Tropica*, **98**, 219.

Bradley, C.A., and Altizer, S. (2005). Parasites hinder monarch butterfly flight: implications for disease spread in migratory hosts. *Ecology Letters*, **8**, 290.

Burand, J.P., Rallis, C.P., and Tan, W. (2004). Horizontal transmission of Hz-2V by virus infected *Helicoverpa zea* moths. *Journal of Invertebrate Pathology*, **85**, 128.

Burand, J.P., Tan, W., Kim, W., Nojima, S., and Roelofs, W. (2005). Infection with the insect virus Hz-2v alters mating behavior and pheromone production in female *Helicoverpa zea* moths. *Journal of Insect Science*, **5**, 6.

Carmo-Sousa, M., Moreno, A., Garzo, E., and Fereres, A. (2014). A non-persistently transmitted-virus induces a pull-push strategy in its aphid vector to optimize transmission and spread. *Virus Research*, **186**, 38.

Chapuisat, M., Oppliger, A., Magliano, P., and Christe, P. (2007). Wood ants use resin to protect themselves against pathogens. *Proceedings of the Royal Society B*, **274**, 2013.

Cremer, S., Armitage, S.A.O., and Schmid-Hempel, P. (2007). Social immunity. *Current Biology*, **17**, R693.

Crosland, M.W.J., Lok, C.M., Wong, T.C., Shakarad, M., and Traniello, J.F. (1997). Division of labour in a lower termite: the majority of tasks are performed by older workers. *Animal Behavior*, **54**, 999–1012.

Curtis, V.A. (2014). Infection-avoidance behaviour in humans and other animals. *Trends in Immunology*, **35**, 457

Damodaram, K.J.P., Ayyasamy, A., and Kempraj, V. (2016). Commensal bacteria aid mate-selection in the fruit fly, *Bactrocera dorsalis*. *Microbial Ecology*, **72**, 725.

de Roode, J.C., and Lefèvre, T. (2012). Behavioral immunity in insects. *Insects*, **3**, 789–820.

de Roode, J.C., Lefèvre, T., and Hunter, M.D. (2013). Self-medication in animals. *Science*, **340**, 150.

Dell, A.I., Bender, J.A., Branson, K., et al. (2014). Automated image-based tracking and its application in ecology. *Trends in Ecology and Evolution*, **29**, 417–28.

Dheilly, N.M., Maure, F., Ravallec, M., et al. (2015). Who is the puppet master? Replication of a parasitic wasp-associated virus correlates with host behaviour manipulation. *Proceedings of the Royal Society B*, **282**, 20142773.

Egnor, S.E.R., and Branson, K. (2016). Computational analysis of behavior. *Annual Review of Neuroscience*, **39**, 217–36.

Elliot, S.L., Blanford, S., and Thomas, M.B. (2002). Host–pathogen interactions in a varying environment: temperature, behavioural fever and fitness. *Proceedings of the Royal Society B*, **269**, 1599–607.

Feener, D.H., and Brown, B. V. (1992). Reduced foraging of *Solenopsis geminata* (Hymenoptera: Formicidae) in the presence of parasitic *Pseudacteon* spp. (Diptera' Phoridae). *Annals of the Entomological Society of America*, **85**, 80.

Fouks, B., and Lattorff, H.M.G. (2011). Recognition and avoidance of contaminated flowers by foraging bumblebees (*Bombus terrestris*). *PLoS One*, **6**(10), e26328.

Gaugler, R., Wang, Y.I., and Campbell, J.F. (1994). Aggressive and evasive behaviors in Popillia japonica (Coleoptera: Scarabaeidae) larvae: defenses against entomopathogenic nematode attack. *Journal of Invertebrate Pathology*, **64**, 193.

Giannoni-Guzmán, M.A., Avalos, A., Perez, J.M., et al. (2014). Measuring individual locomotor rhythms in honey bees, paper wasps and other similar-sized insects. *Journal of Experimental Biology*, **217**, 1307.

Gilestro, G.F. (2012). Video tracking and analysis of sleep in *Drosophila melanogaster*. *Nature Protocols*, **7**, 995.

Goater, T.P., Goater, C.P., and Esch G.W. (2014). *Parasitism: the diversity and ecology of animal parasites*, 2nd edn. Cambridge University Press, Cambridge.

Goldsworthy, G., Opoku-Ware, K., and Mullen, L. (2002). Adipokinetic hormone enhances laminarin and bacterial lipopolysaccharide-induced activation of the prophenoloxidase cascade in the African migratory locust, *Locusta migratoria*. *Journal of Insect Physiology*, **48**, 601.

Grosman, A.H., Janssen, A., De Brito, E.F., et al. (2008). Parasitoid increases survival of its pupae by inducing hosts to fight predators. *PLoS One*, **3**, e2276.

Harabis, F., Dolný, A., Helebrandova, J., and Rusková, T. (2015). Do egg parasitoids increase the tendency of *Lestes sponsa* (Odonata: Lestidae) to oviposit underwater? *European Journal of Entomology*, **112**, 63.

Hart, B.L. (1988). Biological basis of the behavior of sick animals. *Neuroscience & Biobehavioral Reviews*, **12**, 123.

Hart, B.L. (2011). Behavioural defences in animals against pathogens and parasites: parallels with the pillars of medicine in humans. *Philosophical Transactions of the Royal Society B*, **366**, 3406.

Harvey, J.A., Tanaka, T., Kruidhof, M., Vet, L.E.M., and Gols, R. (2011). The 'usurpation hypothesis' revisited: dying caterpillar repels attack from a parasitoid wasp. *Animal Behavior*, **81**, 1281.

Heil, M. (2016). Host manipulation by parasites: cases, patterns, and remaining doubts. *Frontiers in Ecology and Evolution*, **4**, 80.

Heinze, J., and Walter, B. (2010). Moribund ants leave their nests to die in social isolation. *Current Biology*, **20**, 249.

Hohorst, W., and Graefe, G. (1961). Ameisen—obligatorische Zwischenwirte des Lanzettegels (*Dicrocoelium dendriticum*). *Naturwissenschaften*, **48**(7), 229.

Hölldobler, B. (2012). Afterward. In: D. P. Hughes, J. Brodeur, and F. Thomas (Eds), *Host Manipulation by Parasites*, pp. 155–7. Oxford University Press, Oxford.

Huang, J., Wu, S-F., Li, X-H., Adamo, S.A., and Ye, G-Y. (2012). The characterization of a concentration-sensitive α-adrenergic-like octopamine receptor found on insect immune cells and its possible role in mediating stress hormone effects on immune function. *Brain, Behavior, and Immunity*, **26**, 942.

Hunt, V.L., Zhong, W., McClure, C.D., et al. (2016). Cold-seeking behaviour mitigates reproductive losses from fungal infection in *Drosophila*. *Journal of Animal Ecology*, **85**, 178.

Hurd, H. (2003). Manipulation of medically important insect vectors by their parasites. *Annual Review of Entomology*, **48**, 141.

Kacsoh, B.Z., Lynch, Z.R., Mortimer, N.T., and Schlenke, T.A. (2013). Fruit flies medicate offspring after seeing parasites. *Science*, **339**, 947.

Kalsbeek, V., Mullens, B.A., and Jespersen, J.B. (2001). Field studies of entomophthora (Zygomycetes: Entomophthorales)—induced behavioral fever in *Musca domestica* (Diptera: Muscidae) in Denmark. *Biological Control*, **21**, 264.

Kazlauskas, N., Klappenbach, M., Depino, A.M., and Locatelli, F.F. (2016). Sickness Behavior in Honey Bees. *Frontiers in Physiology* 7.

Klopfer, P.H., and Hailman, J.P. (1972). *Function and Evolution of Behavior*. Addison-Wesley Pub. Co., Boston, MA.

Kohmura, H., Hirayama, H., and Ueno, T. (2015). Diving into the water: cues related to the decision-making by an egg parasitoid attacking underwater hosts. *Ethology*, **121**, 168.

Konrad, M., Vyleta, M.L., Theis, F.J., et al. (2012). Social transfer of pathogenic fungus promotes active immunisation in ant colonies. *PLoS Biology*, **10**, e1001300.

Kreuter, K., Bunk, E., Lückemeyer, A., et al. (2012). How the social parasitic bumblebee Bombus bohemicus sneaks into power of reproduction. *Behavioral Ecology and Sociobiology*, **66**, 475.

Lafferty, K.D., and Kuris, A.M. (2012). Ecological consequences of manipulative parasites. In: D.P. Hughes, J. Brodeur, and F. Thomas (Eds), *Host Manipulation by Parasites*, pp. 158–79. Oxford University Press, Oxford.

Laland, K., Wray G.A., and Hoekstra, H.E. (2014). Does evolutionary theory need a rethink? *Nature*, **514**, 161.

Lambardi, D., Dani, F.R., Turillazzi, S., and Boomsma, J.J. (2007). Chemical mimicry in an incipient leaf-cutting ant social parasite. *Behavioral Ecology and Sociobiology*, **61**, 843.

Lee, K.P., Simpson, S.J., and Wilson, K. (2008). Dietary protein-quality influences melanization and immune function in an insect. *Functional Ecology*, **22**, 1052.

Lefèvre, T., Oliver, L., Hunter, M.D., and de Roode, J.C. (2010). Evidence for trans-generational medication in nature. *Ecology Letters*, **13**, 1485–93.

Lopes, P.C. (2014). When is it socially acceptable to feel sick? *Proceedings of the Royal Society B*, **281**, 20140218.

Lopes, P.C. (2017). Why are behavioral and immune traits linked? *Hormones and Behavior*, **88**, 52.

Lusebrink, I., Dettner, K., and Seifert, K. (2008). Stenusine, an antimicrobial agent in the rove beetle genus *Stenus* (Coleoptera, Staphylinidae). *Naturwissenschaften*, **95**, 751.

Malfi, R.L., Davis, S.E., and Roulston, T.H. (2014). Parasitoid fly induces manipulative grave-digging behaviour differentially across its bumblebee hosts. *Animal Behavior*, **92**, 213.

McBride, C.S., and Arguello, J.R. (2007). Five *Drosophila* genomes reveal nonneutral evolution and the signature of host specialization in the chemoreceptor superfamily. *Genetics*, **177**, 1395.

McMenemy, L.S., Hartley, S.E., MacFarlane, S.A, et al. (2012). Raspberry viruses manipulate the behaviour of their insect vectors. *Entomologia Experimentalis et Applicata*, **144**, 56.

Mersch, D.P., Crespi, A., and Keller, L. (2013). Tracking individuals shows spatial fidelity is a key regulator of ant social organization. *Science*, **340**, 1090.

Moore, J. (2013). An overview of parasite-induced behavioral alterations—and some lessons from bats. *Journal of Experimental Biology*, **216**, 11.

Moore, J., and Freehling, M. (2002). Cockroach hosts in thermal gradients suppress parasite development. *Oecologia*, **133**, 261.

Müller, C.B., and Schmid-Hempel, P. (1993). Exploitation of cold temperature as defence against parasitoids in bumblebees. *Nature*, **363**, 65.

Murray, M.J., and Murray, A.B. (1979). Anorexia of infection as a mechanism of host defense. *American Journal of Clinical Nutrition*, **32**, 593.

Neoh, K-B., Yeap, B-K., Tsunoda, K., Yoshimura, T., and Lee, C-Y. (2012). Do termites avoid carcasses? Behavioral responses depend on the nature of the carcasses. *PLoS One*, 7, e36375.

Nielsen, C., Agrawal, A.A., and Hajek, A.E. (2010). Ants defend aphids against lethal disease. *Biology Letters*, **6**(2), 205–8.

Orr, M. (1992). Parasitic flies (Diptera: Phoridae) influence foraging rhythms and caste division of labor in the leafcutter ant, *Atta cephalotes* (Hymenoptera: Formicidae). *Behavioral Ecology and Sociobiology*, **30**, 395.

Parker, B.J., Elderd, B.D., and Dwyer, G. (2010). Host behaviour and exposure risk in an insect-pathogen interaction. *Journal of Animal* Ecology, **79**, 863.

Pfeiffenberger, C., Lear, B.C., Keegan, K.P., and Allada, R. (2010). Locomotor activity level monitoring using the *Drosophila* activity monitoring (DAM) system. *Cold Spring Harbor Protocols*, **11**, pdb.prot5518.

Pie, M.R., Rosengaus, R.B., and Traniello, J.F.A. (2004). Nest architecture, activity pattern, worker density and the dynamics of disease transmission in social insects. *Journal of Theoretical Biology*, **226**, 45.

Ponton, F., Wilson, K., Holmes, A.J., et al. (2013). Integrating nutrition and immunology: a new frontier. *Journal of Insect Physiology*, **59**, 130.

Poulin, R. (1995). 'Adaptive' changes in the behaviour of parasitized animals: a critical review. *International Journal for Parasitology*, **25**, 1371.

Poulin, R. (2010). Parasite manipulation of host behavior: an update and frequently asked questions. In: T.J.R.H. Jane Brockmann, M. Naguib, K. E. Wynne-Edwards, J. C. Mitani, and L. W. Simmons (Eds), *Advances in the Study of Behavior*, pp. 151–86. Academic Press, Cambridge, MA.

Reiser, M. (2009). The ethomics era? *Nature Methods*, **6**, 413.

Rigaud, T., Perrot-Minnot, M.J., and Brown, M.J. (2010). Parasite and host assemblages: embracing the reality will improve our knowledge of parasite transmission and virulence. *Proceedings of the Royal Society B*, **277**, 3693.

Rosengaus, R.B., James, L-T., Hartke, T.R., and Brent, C.S. (2011). Mate preference and disease risk in *Zootermopsis angusticollis* (Isoptera: Termopsidae). *Environmental Entomology*, **40**, 1554.

Rund, S.S.C., O'Donnell, A.J., Gentile, J.E., and Reece, S.E. (2016). Daily rhythms in mosquitoes and their consequences for malaria transmission. *Insects*, **7**, 14.

Sadek, M.M., Hansson, B.S., and Anderson, P. (2010). Does risk of egg parasitism affect choice of oviposition sites by a moth? A field and laboratory study. *Basic and Applied Ecology*, **11**, 135.

Sato, T., Egusa, T., Fukushima, K., et al. (2012). Nematomorph parasites indirectly alter the food web and ecosystem function of streams through behavioural manipulation of their cricket hosts. *Ecology Letters*, **15**, 786.

Satterfield, D.A., Maerz, J.C., and Altizer, S. (2015). Loss of migratory behaviour increases infection risk for a butterfly host. *Proceedings of the Royal Society B*, **282**, 20141734.

Schmid-Hempel, P. (2011). *Evolutionary Parasitology: the Integrated Study of Infections, Immunology, Ecology, and Genetics*. Oxford University Press, Oxford.

Schutgens, M., Cook, B., Gilbert, F., and Behnke, J.M. (2015). Behavioural changes in the flour beetle *Tribolium confusum* infected with the spirurid nematode *Protospirura muricola*. *Journal of Helminthology*, **89**, 68.

Shirasu-Hiza, M.M., Dionne, M.S., Pham, L.N., Ayres, J.S., and Schneider, D.S. (2007). Interactions between circadian rhythm and immunity in *Drosophila melanogaster*. *Current Biology*, **17**, R353.

Simon, A.F., Chou, M-T., Salazar, E.D., et al. (2012). A simple assay to study social behavior in *Drosophila*: measurement of social space within a group. *Genes, Brain and Behavior*, **11**, 243.

Singer, M.S., Mason, P.A., and Smilanich, A.M. (2014). Ecological immunology mediated by diet in herbivorous insects. *Integrative and Comparative Biology*, **54**, 913.

Siva-Jothy, M.T. (1999). Male wing pigmentation may affect reproductive success via female choice in a calopterygid damselfly Zygoptera). *Behaviour*, **136**, 1365.

Stahlschmidt, Z.R., and Adamo, S.A. (2013). Context dependency and generality of fever in insects. *Naturwissenschaften*, **100**, 691.

Starks, P.T., Blackie, C.A., and Seeley, T.D. (2000). Fever in honeybee colonies. *Naturwissenschaften*, **87**, 229.

Stensmyr, M.C., Dweck, H.K.M., Farhan, A., et al. (2012). A conserved dedicated olfactory circuit for detecting harmful microbes in *Drosophila*. *Cell*, **151**, 1345.

Sullivan, K., Fairn, E., and Adamo, S.A. (2016). Sickness behaviour in the cricket *Gryllus texensis*: comparison with animals across phyla. *Behavioral Processes*, **128**, 134.

Tasin, M., Knudsen, G.K., and Pertot, I. (2012). Smelling a diseased host: grapevine moth responses to healthy and fungus-infected grapes. *Animal Behavior*, **83**, 555.

Thomas, F., Maure, F., Brodeur, J., et al. (2011). The cost of a bodyguard. *Biology Letters*, **7**, 843.

Thomas, F., Rigaud, T., and Brodeur, J. (2012). Evolutionary routes leading to host manipulation by parasites. In: D.P. Hughes, J. Brodeur, and F. Thomas, (Eds), *Host*

Manipulation by Parasites, pp. 16–35. Oxford University Press, Oxford.

Thomas, M.B., and Blanford, S. (2003). Thermal biology in insect–parasite interactions. *Trends in Ecology and Evolution*, **18**, 344.

Tinbergen, N. (1963). On aims and methods of ethology. *Zeitschrift für Tierpsychologie*, **20**, 410.

Tregenza, T., Simmons, L.W., Wedell, N., and Zuk, M. (2006). Female preference for male courtship song and its role as a signal of immune function and condition. *Animal Behavior*, **72**, 809.

Turillazzi, S., Mastrobuoni, G., Dani, F.R., et al. (2006). Dominulin A and B: two new antibacterial peptides identified on the cuticle and in the venom of the social paper wasp *Polistes dominulus* using MALDI-TOF, MALDI-TOF/TOF, and ESI-Ion Trap. *Journal of the American Society for Mass Spectrometry*, **17**, 376.

Vale, P.F., and Jardine, M.D. (2015). Sex-specific behavioural symptoms of viral gut infection and *Wolbachia* in *Drosophila melanogaster*. *Journal of Insect Physiology*, **82**, 28.

Vale, P.F., and Jardine, M.D. (2017). Infection avoidance behavior: viral exposure reduces the motivation to forage in female Drosophila melanogaster. *Fly*, **11**(1), 3–9.

Valentine, B.D. (2007). Mutual grooming in cucujoid beetles (Coleoptera: Silvanidae). *Insecta Mundi* **2007** (0001–0008), 1–3.

Weinstein, S.B., and Kuris, A.M. (2016). Independent origins of parasitism in Animalia. *Biology Letters*, **12**, 20160324.

Zhukovskaya, M., Yanagawa, A., and Forschler, B. (2013). Grooming behavior as a mechanism of insect disease defense. *Insects*, **4**, 609.

7.5.3 Oral Bacterial Infection and Shedding in *Drosophila melanogaster*

Siva-Jothy, J. A.; Prakash, A.; Vasanthakrishnan, R. B.; Monteith, K. M.; and Vale, P. F (2018) Oral Bacterial Infection and Shedding in *Drosophila melanogaster*. *JoVE (Journal of Visualized Experiments)*, (135): e57676–e57676.

Video is available at:

https://www.jove.com/video/57676/oral-bacterial-infection-and-shedding-in-drosophila-melanogaster

or

https://tinyurl.com/y2efmye5

Video Article

Oral Bacterial Infection and Shedding in *Drosophila melanogaster*

Jonathon A. Siva-Jothy[1], Arun Prakash[1], Radhakrishnan B. Vasanthakrishnan[2], Katy M. Monteith[1], Pedro F. Vale[1,3]

[1]Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh

[2]IGDR - CNRS UMR 6290

[3]Centre for Immunity, Infection and Evolution, University of Edinburgh

Correspondence to: Pedro F. Vale at pedro.vale@ed.ac.uk

URL: https://www.jove.com/video/57676
DOI: doi:10.3791/57676

Keywords: Biology, Issue 135, Infection, immunity, *Drosophila*, oral infection, bacterial shedding, Relish, Dcy

Date Published: 5/31/2018

Citation: Siva-Jothy, J.A., Prakash, A., Vasanthakrishnan, R.B., Monteith, K.M., Vale, P.F. Oral Bacterial Infection and Shedding in *Drosophila melanogaster*. *J. Vis. Exp.* (135), e57676, doi:10.3791/57676 (2018).

Abstract

The fruit fly *Drosophila melanogaster* is one of the best developed model systems of infection and innate immunity. While most work has focused on systemic infections, there has been a recent increase of interest in the mechanisms of gut immunocompetence to pathogens, which require methods to orally infect flies. Here we present a protocol to orally expose individual flies to an opportunistic bacterial pathogen (*Pseudomonas aeruginosa*) and a natural bacterial pathogen of *D. melanogaster* (*Pseudomonas entomophila*). The goal of this protocol is to provide a robust method to expose male and female flies to these pathogens. We provide representative results showing survival phenotypes, microbe loads, and bacterial shedding, which is relevant for the study of heterogeneity in pathogen transmission. Finally, we confirm that *Dcy* mutants (lacking the protective peritrophic matrix in the gut epithelium) and Relish mutants (lacking a functional immune deficiency (IMD) pathway), show increased susceptibility to bacterial oral infection. This protocol, therefore, describes a robust method to infect flies using the oral route of infection, which can be extended to the study of a variety genetic and environmental sources of variation in gut infection outcomes and bacterial transmission.

Video Link

The video component of this article can be found at https://www.jove.com/video/57676/

Introduction

The fruit fly (also known as the vinegar fly), *D. melanogaster*, has been extensively used as a model organism for infection and immunity against a variety of pathogens[1,2]. This work has offered fundamental insights into the physiological consequences of infection and was also pioneering in unraveling the molecular pathways underlying the host immune response against parasitoid, bacterial, fungal, and viral infections. This knowledge is not only useful to understand the innate immune response of insects and other invertebrates, but because many of the immune mechanisms are evolutionarily conserved between insects and mammals, *Drosophila* has also spurred the discovery of major immune mechanisms in mammals, including humans[3].

Most work on *Drosophila* infection and immunity has focused on systemic infections, using inoculation methods that deliver pathogens directly into the body of the insect by pricking or injection[4,5,6]. The advantage of these methods in allowing the delivery of a controlled infectious dose is clear and supported by a large body of work on systemic infections. However, many naturally occurring bacterial pathogens of *D. melanogaster* are acquired through feeding on decomposing organic matter where gut immunocompetence plays a significant role in host defence[7,8,9,10,11,12,13,14,15]. Experiments that employ systemic infections bypass these defenses, and, therefore, provide an altogether different picture of how insects mount defenses against natural pathogens. This is especially relevant if the aim of the work is to test predictions about the ecology and evolution of infection, where the use of natural pathogens and routes of infection is important[16,17]. Recent work has highlighted how the route taken by pathogens significantly affects disease outcome[18,19], elicits distinct immune pathways[20,21], can determine the protective effect of inherited endosymbionts[16], and may even play an important role in the evolution of host defenses[17].

Another reason to employ oral routes of infection is that it allows the investigation of the variation in pathogen transmission by measuring bacterial shedding during fecal excretion following oral infection[22,23,24]. Understanding the sources of host heterogeneity in disease transmission is challenging in natural populations[25,26], but measuring components of transmission, such as pathogen shedding, under controlled laboratory conditions offers a useful alternative approach[27]. By feeding flies bacteria and measuring bacterial shedding under a variety of genetic and environmental contexts in controlled experimental conditions, it is possible to identify sources of variation in transmission among hosts.

Here, we describe a protocol for orally infecting *D. melanogaster* with bacterial pathogens, and for quantifying the bacterial growth and shedding that follows (**Figure 1**). We describe this protocol on two *Pseudomonas* bacteria: a virulent strain of the opportunistic pathogen *P. aeruginosa* (PA14), and a less virulent strain of the natural fly pathogen *P. entomophila*. Pseudomonads are common gram-negative bacteria with a broad host range, infecting insects, nematodes, plants, and vertebrates, and are found in most environments[4,6]. Enteric infection of *Drosophila* by *P. aeruginosa* and *P. entomophila* results in pathology to intestinal epithelia[12,13,14,15,28]. While we focus on these two bacterial pathogens, the methods described here can in principle be applied to any bacterial pathogen of interest with minor modifications. Following oral exposure, we measure post-infection survival, and measure the microbe load within individual flies and the viable microbes shed into the environment,

expressed in colony forming units (CFUs). Finally, because gut immunocompetence results from a combination of epithelial barrier and humoral responses, we also measure the survival of fly lines where these defenses are disrupted. Specifically, Drosocrystallin (*Dcy)* mutants have been previously shown to be more susceptible to oral bacterial infection due to a depleted peritrophic matrix in the gut[29]. We also measure survival in a Relish (*Rel*) mutant which is impeded from producing antimicrobial peptides against Gram-negative bacteria via the IMD pathway[30].

Protocol

1. Maintain Flies

1. Maintain flies in 23 mL plastic vials containing 7 mL of freshly made Lewis medium (modified from reference[31]; 1 L triple distilled H_2O, 6.1 g agar, 93.6 g brown sugar, 68 g maize, 18.7 g instant yeast, 15 mL Tegosept anti-fungal agent) in incubators at 25 ± 1 °C, in a 12 h:12 h light:dark cycle with ~60% humidity. Plug the vials with non-absorbent cotton wool.
2. After every 14 days, transfer 20–30 adults to a new food vial, with instant, dry yeast added to the surface, for 2–3 days to allow egg-laying to occur. After this time period, ensure that the eggs are visible on the surface of the food. Remove adult flies.
   NOTE: This keeps flies in the vials as single generation, age-matched populations.
3. Leave the eggs to develop.
   NOTE: At 25 °C, adult flies start to eclose from pupae on day 11 and continue over days 12–14.

2. Prepare Experimental Flies

1. Collect the eggs of the parent generation in a population/embryo collection cage on a 75 mL apple-agar plate (1 L triple distilled H_2O, 30 g agar, 33 g sucrose, 330 mL apple juice, 7 mL Tegosept anti-fungal agent) with a yeast paste spread (mix dry yeast with water to a peanut butter-like consistency). Add water-soaked cotton wool to the cage to provide moisture.
   NOTE: To avoid confounding effects caused by differences in larval rearing density, it is important that experimental flies in different vials are reared in similar densities. The above step is performed to avoid confounding effects.
2. Incubate for 24 h at 25 °C in a 12 h:12 h light:dark cycle until egg-laying has occurred. If there are too few eggs after 24 h, provide a longer habituation period. Replace apple-agar plates and allow egg-laying to occur for a further 24 h.
3. Take egg-laden apple-agar plates from the population cage. Remove the remaining yeast paste and any dead flies from the agar's surface.
4. Submerge the agar in 20 mL of 1x phosphate-buffered saline (PBS) and gently dislodge the eggs from the apple-agar with a fine paintbrush. While suspended in PBS, transfer the eggs to a 50 mL centrifuge tube and leave for 5 min so the eggs sink to the bottom.
   NOTE: Most eggs are found on the outer edge of the agar.
5. Remove by cutting the bottom 4 mm of a p1000 filtered pipette tip and use the pipette tip to draw 1 mL of solution, taken from the bottom of the 50 mL centrifuge tube. Transfer this to a 1.5 mL microcentrifuge tube and allow it to settle.
   NOTE: When pipetting up eggs, snap-releasing the plunger is more efficient than a gentle release.
6. Remove by cutting the bottom 4 mm of a p20 filtered pipette tip. Set the pipette to a desired volume and draw from the bottom of the microcentrifuge tube.
   NOTE: With practice, a volume of 5 µL contains roughly 100 eggs.
7. Dispense the collected eggs onto the food and leave them to develop for the required amount of time.

3. Bacterial Culture

1. To grow *P. entomophila* and *P. aeruginosa* cultures, inoculate 10 mL of Luria-Bertani (LB) broth with 100 µL of a frozen bacterial stock at 30 °C (*P. entomophila)* and 37 °C (*P. aeruginosa*), respectively. Shake at 150 rpm overnight. Ensure that the bacterial culture reaches the saturation phase.
2. To ensure the bacteria used for inoculating the flies are in the exponential phase and rapidly replicating, inoculate the overnight culture into a new subculture, of a desired volume, the following morning. Ensure that the pre-inoculum is 10% of the total volume of the subculture culture.
   NOTE: Oral infection requires high . It is therefore necessary to grow a substantial volume of bacterial culture so that enough inoculation culture can be produced for the desired dose and experimental size. Calculate how much subculture is needed to produce the required infectious doses using the equation $M_sV_s = M_iV_i$, where M represents a culture's optical density measured at 600 nm (OD_{600}) value and V represents its volume. Subscript letters refer to whether the culture is used as a subculture (s) or an infectious dose (i).
3. Grow this subculture in a 2 L conical flask in a volume such that the subculture's surface falls (at most) just above the beginning of the flask's slope. Do not fill above this mark as it will stunt the growth of bacteria.
4. Ensure the bacteria in this subculture are in the exponential growth phase by measuring the OD every 30 min.
   NOTE: This occurs after 3–5 h, where the subculture reaches an OD_{600} between 0.6–0.8.
5. Pour equal volumes of this subculture across 50 mL centrifuge tubes and spin the subculture at 2,500 x g for 15 min at 4 °C to pellet the bacteria. Once pelleted, remove and then spin the supernatant again at the above conditions to confirm the removal of the vast majority of bacteria.
   NOTE: A pellet of negligible size (smaller than 1 mm in height) confirms this.
6. Combine the bacterial pellets of the separate tubes by re-suspending them in 5 mL of subculture supernatant and recombining these solutions in a single 50 mL tube. Spin this concentrated culture at 2,500 x g for 15 min at 4 °C to pellet the bacteria.
7. Remove the supernatant and re-suspend the final bacteria pellet in 5% sucrose water solution. Check the OD and adjust to the desired infectious dose (OD_{600} = 100 for *P. entomophila*[8] and OD_{600} = 25 for *P. aeruginosa*[16,28]), by re-suspending the pellets in 5% sucrose water solution to the required volume.
   NOTE: The amount of 5% sucrose water solution to be added can be calculated using the equation in step 3.2.1 ($M_sV_s = M_iV_i$).

4. Orally Infecting Flies

1. To ensure oral infection, starve the flies for 2–4 h before exposure to bacteria by transferring the flies to standard agar vials (1 L triple distilled H_2O, 20 g agar, 84 g brown sugar, 7 mL Tegosept anti-fungal agent).
2. Prepare infection vials while flies are being starved. Make a *Pseudomonas* infection vial by pipetting 500 µL of standard sugar agar into the lid of a 7 mL sample tube and leave it to dry. Place a disc of filter paper in the lid and pipette 100 µL of bacterial culture directly onto the filter disc. For control infections, replace the bacterial culture with the same volume of 5% sucrose water solution on the filter paper.
3. Add single flies to the sample tube and leave for 18–24 h.
4. To confirm oral infection, first surface-sterilize the flies immediately after bacterial exposure, by placing them in 100 µL of 70% ethanol for 20–30 s. Remove the ethanol and add 100 µL of triple distilled water for 20–30 s before removing the water. Add 100 µL of 1x PBS and homogenize the fly.
5. Transfer the homogenate to the top row of a 96-well plate and add 90 µL of 1x PBS to every well below.
6. Serially dilute this sample to distinguish a range of CFU values. Take 10 µL of the homogenate in the top well and add this to the well below. Repeat this step with the second well, transferring 10 µL to the third well, and so on, for as many serial dilutions as required.
   NOTE: It important that new pipette tips are used for each set of dilutions.
7. Plate the serial dilutions on an LB nutrient agar plate in 5 µL droplets, to ensure all droplets remain discrete.
8. Incubate the LB Agar plates overnight at 30 °C and 37 °C for *P. entomophila* and *P. aeruginosa*, respectively and count visible CFUs.
   NOTE: While *Drosophila* gut microbes require distinct anaerobic growth conditions, selective medium, for example *Pseudomonas* Isolation Medium (PIM), may be used to make sure only *Pseudomonas* CFUs are counted.
9. Calculate the number of CFUs per fly by counting the number of colonies present at the serial dilution where 10–60 CFUs are clearly visible. Then multiply by the dilution factor present to calculate the number of bacteria per fly.
10. Perform statistical analysis. Where necessary, transform the CFUs per fly to a normal distribution. Do this by log-transformation. Once transformed, use Generalized Linear Models (GLMs)[30,31,32] to test how treatment groups differ in CFUs per fly (using commonly available statistical software packages such as R[33]).
    NOTE: The remaining fly homogenate can be used for measuring gene expression through quantitative reverse transcription PCR (RT-qPCR) analysis. Fix the homogenate in 50 µL of RNA isolation reagent, extract RNA, and quantify specific immune gene titers by RT-qPCR (see *e.g.*, Gupta and Vale[16] for a detailed protocol). The expression of specific immune gene transcripts should be normalized to the transcript levels of a housekeeping gene (*i.e.*, rp49) and expressed as a fold change relative to control flies using the $2^{-\Delta\Delta Ct}$ method[31,32,33,34].

5. Recording Survivorship Following Infection

1. Infect flies orally as described in step 4.2.
2. Transfer the infected or control flies from their respective infection vials into standard Lewis vials and keep in an incubator at 25 °C in a 12 h:12 h light-dark cycle (or desired conditions). Keep flies until they are dead.
3. Count the number of living or dead flies in each vial every day, or as often as required.
4. Transfer the flies to new vials every 5 days to avoid the flies getting stuck in the food.
5. Present these data as Kaplan-Meier (KM) survival curves or Mean ± SE proportional survival plots. To analyze the effect of several factors and/or their respective interactions with one another use a statistical package (for example, the package "survival" in R[33]) to run a survival analysis such as the Cox Proportional Hazards model[35].

6. Measuring Bacterial Load

1. At the desired time point, transfer a single infected fly to a sterile 1.5 mL microcentrifuge tube.
2. Surface sterilize the flies as described in step 4.4.
3. Homogenize the fly and quantify the bacterial load using the protocol described in steps 4.5–4.10.

7. Measure Bacterial Shedding

1. Measure the shedding alongside the internal load.
2. After infection, transfer single flies to 1.5 mL microcentrifuge tubes containing ~50 µL of Lewis medium for 24 h.
3. Remove the flies for the internal load measurement (see step 6) and wash the tubes with 100 µL of 1x PBS by vortexing heavily for 3 s.
4. Measure the CFUs in this wash by plating on LB nutrient agar using the same protocol as described in steps 4.6–4.8.
5. After infection, transfer single flies to 1.5 mL microcentrifuge tubes containing ~50 µL of Lewis medium for 24 h.
6. Transfer the flies to new microcentrifuge tubes containing ~50 µL of Lewis medium for a further 24 h. Wash the contaminated tubes with 100 µL of 1x PBS by vortexing heavily for 3 s.
7. Measure the CFUs in this wash by plating on LB nutrient agar using the same protocol described in steps 4.6–4.8.
8. Repeat steps 7.2 and 7.3 and record fly mortality at every transfer.

Representative Results

Here, we present illustrative results from experiments where *D. melanogaster* was orally infected with *P. aeruginosa* or *P. entomophila*. **Figure 2** demonstrates the successful oral infection of flies following a 12 h or 24 h exposure period to bacterial cultures of OD_{600} = 25 and 100 for *P. aeruginosa* (**Figure 2A**) and *P. entomophila* (**Figure 2B, C**), respectively. **Figure 2B** illustrates the importance of using a more concentrated culture of *P. entomophila*, shown by the increase in bacterial load when flies are exposed to bacterial cultures of greater optical density. Male and female Oregon R (OreR) flies clear *P. aeruginosa* infection at the same rate (**Figure 3**) and shed the same number of *P. aeruginosa* CFUs (**Figure 4A**). When infected with *P. entomophila* however, male and female OreR flies differ in the number of bacteria shed, in a manner that changes over time (**Figure 4B**). Males and females die from *P. aeruginosa* (**Figure 5A**) and *P. entomophila* (**Figure 5B**) at different rates. We also see that *Dcy* mutants (which lack the protective peritrophic matrix in the gut epithelium) and Relish mutants (which lack a functional IMD immune pathway), show decreased survival following *P. entomophila* and *P. aeruginosa* oral infection (**Figure 5C**).

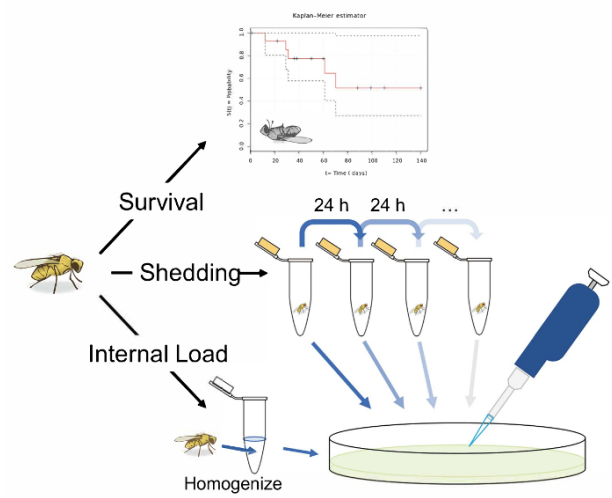


Figure 1: Schematic overview of protocols for measuring survival, shedding, and internal bacterial load following oral infection in *Drosophila melanogaster*. An illustration of 3 potential experiments following the oral infection of *D. melanogaster*. Measure the 'survival' by transferring single flies to vials and recording their infected lifespan. Measure 'shedding' by transferring single flies to 1.5 mL microcentrifuge tubes with 50 µL of Lewis medium in the cap. After 24 h in the tube, remove the fly and vortex the tube with 100 µL of 1x PBS. Remove and plate this solution on LB nutrient agar to calculate the bacterial shedding. Measure the shedding in the same fly longitudinally, by transferring flies to fresh tubes with Lewis medium in the cap after 24 h, and washing and plating the now contaminated tube. A fly's 'internal load' can be measured by taking an infected fly, surface sterilizing it, and homogenizing it before finally plating the homogenate on LB nutrient agar. This can be performed after shedding has been measured to calculate how the 'internal load' and shedding correlate. The fly illustration used in this figure was originally drawn by B. Nuhanen[36]. The authors have modified it to accompany the example Kaplan-Meier curve which is taken from Wikimedia Commons[37]. All other illustrations are original. Please click here to view a larger version of this figure.

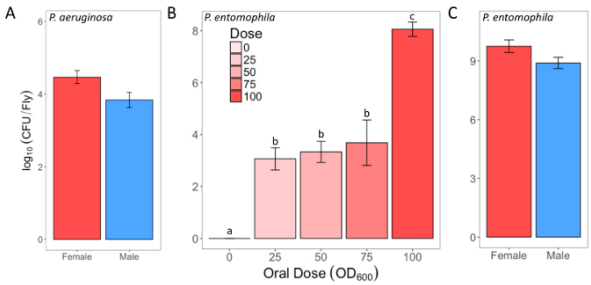


Figure 2: Infectious dose of bacteria following oral infection. (A) Infectious dose of male and female Oregon-R flies following exposure to a *P. aeruginosa* culture (OD_{600} = 25) for 12 h. The mean and SE were calculated from 3 males and 3 females. **(B)** Infectious dose of outcrossed wild-type females following exposure to one of four *P. entomophila* cultures (OD_{600} = 100, 75, 50, and 25) or control 5% sucrose solution for 24 h. The statistical difference of ($F_{3,76}$ = 18.567, p <0.001) in the infectious dose between exposure treatments is denoted by differing letters above bars. The means were calculated from 5 flies for the OD_{600} = 0 dose, and 18-20 for all other doses. **(C)** The infectious dose of male and female Oregon-R flies following exposure to *P. entomophila* culture (OD_{600} = 100) for 24 h. The mean and SE were calculated from 20 males and 20 females. Please click here to view a larger version of this figure.

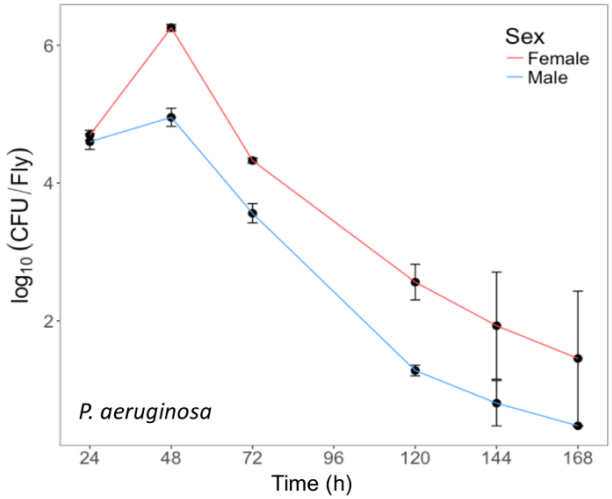


Figure 3: Internal *P. aeruginosa* load in flies after oral infection. Mean ± SE bacterial load of male and female Oregon-R flies following oral infection with *P. aeruginosa* (OD_{600} = 25) up to 168 h post-infection. The mean and SE of each time point are calculated from 3 individuals. A fly's internal bacterial load significantly changes over time (p <0.001). Please click here to view a larger version of this figure.

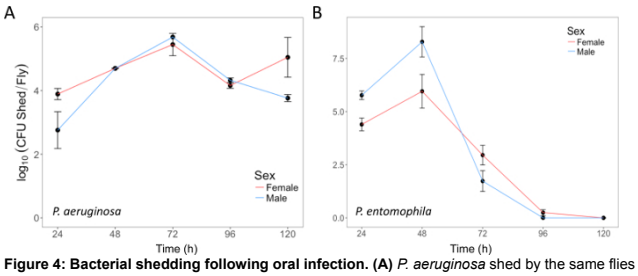


Figure 4: Bacterial shedding following oral infection. (A) *P. aeruginosa* shed by the same flies used in **Figure 3**, up to 120 h post-infection. The mean and SE were calculated from 3 males and 3 females. **(B)** *P. entomophila* shed by male and female Oregon-R flies following oral infection with *P. entomophila* (OD_{600} = 100) up to 120 h post-infection. The mean and SE were calculated from 34 males and 38 females. For both *P. aeruginosa* and *P. entomophila*, the number of CFUs shed by a fly significantly changes over time (p <0.001). Please click here to view a larger version of this figure.

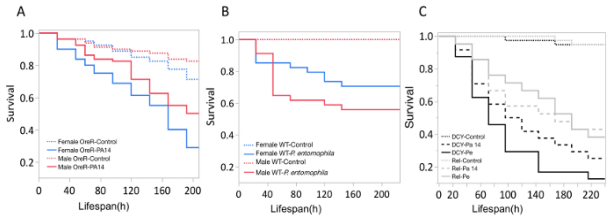


Figure 5: Survival of flies following bacterial oral infection. Kaplan-Meier (KM) survival curves of **(A)** Oregon-R male and female flies following oral infection with *P. aeruginosa* (OD_{600} = 25) or control 5% sucrose solution. The KM survival curve was calculated from 4 vials of 20 flies per treatment group. **(B)** OreR male and female flies after oral infection with *P. entomophila* (OD_{600} = 100). The KM survival curve was calculated from 4 single control flies and 34 infected flies for both males and females. **(C)** Immune mutants: *Dcy* (Drosocrystallin-peritrophic matrix mutant) and *Rel* (Relish-IMD mutant), exposed to *P. entomophila* (Pe), *P. aeruginosa* (Pa14), or a control 5% sucrose solution. All infected groups die significantly faster than the control flies (p <0.001). Please click here to view a larger version of this figure.

Discussion

We present a protocol for reliably orally infecting *D. melanogaster* with bacterial pathogens. We focus on *P. aeruginosa* and *P. entomophila*, but this protocol can easily be adapted to enable infection of other bacterial species, *e.g.*, *Serratia marcescens*[7]. Key aspects of this protocol will vary between bacterial species. Accordingly, the most efficient infectious dose, corresponding virulence, and host genotype susceptibility should all be considered and ideally tested in pilot studies. Exposing flies to bacterial cultures of a range of optical densities and measuring their infectious dose and survival is an appropriate starting point when working with new bacterial species or fly lines.

Protocol steps such as fly starvation prior to feeding and re-suspending bacterial pellets in 5% sucrose solution are commonplace in oral infection and increase the reliability of bacterial infection during exposure[7,8,9,10]. However, it is important to note that during exposure, flies essentially live on a surface of bacterial culture. In the process of walking on this culture, bacteria will become lodged on the fly's surface, especially on the cuticle or around the bristles[24]. These epicuticular bacteria, do not reflect a successful enteric infection but would still be detected by the fly homogenization and plating. To reduce the potential for false positives, it is essential to surface sterilize flies through immersion in 70% ethanol for up to 1 min.

When considering bacterial shedding rates, oral infection is essential. The number of pathogens a host releases into the environment is often difficult to measure and the internal load is often taken as a proxy for the severity of infection and therefore transmission[26,27]. Measuring bacterial load alongside bacterial shedding allows an examination of the relationship between these two important components of disease severity and spread[38]. One limitation of the method presented is that assaying the internal bacterial load of flies requires destructive sampling. This makes it difficult to investigate longitudinal trends of pathogen growth and clearance within the same individual. However, it is possible to overcome this limitation by destructively sampling cohorts of individuals at different stages of infection, under the assumption that the average microbe load in each cohort reflects the longitudinal pathogen dynamics within any given individual. Bacterial shedding does not suffer from the same limitations, and we offer examples of how shedding can be quantified in a cross-sectional sample, or longitudinally to investigate how shedding changes within an individual over time.

Many host and pathogen traits jointly determine an individual's propensity to transmit disease[25,26,39]. While the significance of these traits likely varies between host-pathogen systems, shedding is likely a major determinant of fecal-oral transmission. The ability to measure bacterial shedding opens the opportunity to test this assumption. Having characterized host-pathogen dynamics in a desired panel of fly lines,

experimenters could orally infect individuals, and place them alongside uninfected, susceptible hosts during their infectious periods. These 'recipient' flies could then be assayed for internal bacterial load at various time points as a way of directly measuring transmission.

Acknowledgements

This work was supported by a strategic award from the Wellcome Trust for the Centre for Immunity, Infection and Evolution (http://ciie.bio.ed.ac.uk; grant reference no. 095831). PFV was supported by a Branco Weiss fellowship (https://brancoweissfellowship.org/) and a Chancellor's Fellowship (School of Biological Sciences, University of Edinburgh); JASJ was supported by a NERC E3 DTP PhD studentship.

References

1. Hoffmann, J.A. The immune response of Drosophila. *Nature.* **426** (6962), 33-38 (2003).
2. Buchon, N., Silverman, N., Cherry, S. Immunity in Drosophila melanogaster - from microbial recognition to whole-organism physiology. *Nat Rev Immunol.* **14** (12), 796-810 (2014).
3. Bergman, P., Seyedoleslami Esfahani, S., Engström, Y. Chapter Two - Drosophila as a Model for Human Diseases-Focus on Innate Immunity in Barrier Epithelia. *Curr Top Dev Biol.* **121**, 29-81 (2017).
4. Apidianakis, Y., Rahme, L.G. Drosophila melanogaster as a model host for studying Pseudomonas aeruginosa infection. *Nat Protoc.* **4** (9), 1285-1294 (2009).
5. Khalil, S., Jacobson, E., Chambers, M.C., Lazzaro, B.P. Systemic Bacterial Infection and Immune Defense Phenotypes in Drosophila Melanogaster. *JoVE J Vis Exp.* (99), e52613-e52613 (2015).
6. Neyen, C., Bretscher, A.J., Binggeli, O., Lemaitre, B. Methods to study Drosophila immunity. *Methods San Diego Calif.* **68** (1), 116-128 (2014).
7. Nehme, N.T. *et al.* A Model of Bacterial Intestinal Infections in Drosophila melanogaster. *PLOS Pathog.* **3** (11), e173 (2007).
8. Bou Sleiman, M.S., Osman, D., Massouras, A., Hoffmann, A.A., Lemaitre, B., Deplancke, B. Genetic, molecular and physiological basis of variation in Drosophila gut immunocompetence. *Nat Commun.* **6**, 7829 (2015).
9. Buchon, N., Broderick, N.A., Lemaitre, B. Gut homeostasis in a microbial world: insights from Drosophila melanogaster. *Nat Rev Microbiol.* **11** (9), 615-626 (2013).
10. Kuraishi, T., Hori, A., Kurata, S. Host-microbe interactions in the gut of Drosophila melanogaster. *Front Physiol.* **4** (2013).
11. Ha, E.-M., Oh, C.-T., Bae, Y.S., Lee, W.-J. A Direct Role for Dual Oxidase in Drosophila Gut Immunity. *Science.* **310** (5749), 847-850 (2005).
12. Limmer, S. *et al.* Pseudomonas aeruginosa RhlR is required to neutralize the cellular immune response in a Drosophila melanogaster oral infection model. *Proc Natl Acad Sci U S A.* **108** (42), 17378-17383 (2011).
13. Apidianakis, Y., Pitsouli, C., Perrimon, N., Rahme, L. Synergy between bacterial infection and genetic predisposition in intestinal dysplasia. *Proc Natl Acad Sci U S A.* **106** (49), 20883-20888 (2009).
14. Vodovar, N. *et al.* Drosophila host defense after oral infection by an entomopathogenic Pseudomonas species. *Proc Natl Acad Sci U S A.* **102** (32), 11414-11419 (2005).
15. Chugani, S.A., Whiteley, M., Lee, K.M., D'Argenio, D., Manoil, C., Greenberg, E.P. QscR, a modulator of quorum-sensing signal synthesis and virulence in Pseudomonas aeruginosa. *Proc Natl Acad Sci.* **98** (5), 2752-2757 (2001).
16. Gupta, V., Vasanthakrishnan, R.B., Siva-Jothy, J., Monteith, K.M., Brown, S.P., Vale, P.F. The route of infection determines Wolbachia antibacterial protection in Drosophila. *Proc R Soc B.* **284** (1856), 20170809 (2017).
17. Martins, N.E., Faria, V.G., Teixeira, L., Magalhães, S., Sucena, É. Host Adaptation Is Contingent upon the Infection Route Taken by Pathogens. *PLoS Pathog.* **9** (9) (2013).
18. Chambers, M.C., Jacobson, E., Khalil, S., Lazzaro, B.P. Thorax Injury Lowers Resistance to Infection in Drosophila melanogaster. *Infect Immun.* **82** (10), 4380-4389 (2014).
19. Gupta, V., Stewart, C., Rund, S.S.., Monteith, K., Vale, P.F. Costs and benefits of sub-lethal Drosophila C virus infection. *J Evol Biol.* (2017).
20. Ferreira, Á.G., Naylor, H., Esteves, S.S., Pais, I.S., Martins, N.E., Teixeira, L. The Toll-Dorsal Pathway Is Required for Resistance to Viral Oral Infection in Drosophila. *PLoS Pathog.* **10** (12) (2014).
21. Buchon, N., Broderick, N.A., Poidevin, M., Pradervand, S., Lemaitre, B. Drosophila Intestinal Response to Bacterial Infection: Activation of Host Defense and Stem Cell Proliferation. *Cell Host Microbe.* **5** (2), 200-211 (2009).
22. Wayland, M.T. *et al.* Spotting the differences: Probing host/microbiota interactions with a dedicated software tool for the analysis of faecal outputs in Drosophila. *J Insect Physiol.* **69**, 126-135 (2014).
23. Hori, A., Kurata, S., Kuraishi, T. Unexpected role of the IMD pathway in Drosophila gut defense against Staphylococcus aureus. *Biochem Biophys Res Commun.* **495** (1), 395-400 (2018).
24. Ren, C., Webster, P., Finkel, S.E., Tower, J. Increased Internal and External Bacterial Load during Drosophila Aging without Life-Span Trade-Off. *Cell Metab.* **6** (2), 144-152 (2007).
25. Ezenwa, V.O. *et al.* Host behaviour-parasite feedback: an essential link between animal behaviour and disease ecology. *Proc R Soc B.* **283** (1828), 20153078 (2016).
26. McCallum, H. *et al.* Breaking beta: deconstructing the parasite transmission function. *Phil Trans R Soc B.* **372** (1719), 20160084 (2017).
27. Vale, P.F., Choisy, M., Little, T.J. Host nutrition alters the variance in parasite transmission potential. *Biol Lett.* **9** (2), 20121145 (2013).
28. Mulcahy, H., Sibley, C.D., Surette, M.G., Lewenza, S. Drosophila melanogaster as an Animal Model for the Study of Pseudomonas aeruginosa Biofilm Infections In Vivo. *PLoS Pathog.* **7** (10), e1002299 (2011).
29. Kuraishi, T., Binggeli, O., Opota, O., Buchon, N., Lemaitre, B. Genetic evidence for a protective role of the peritrophic matrix against intestinal bacterial infection in Drosophila melanogaster. *Proc Natl Acad Sci.* **108** (38), 15966-15971 (2011).
30. Myllymäki, H., Valanne, S., Rämet, M. The Drosophila Imd Signaling Pathway. *J Immunol.* **192** (8), 3455-3462 (2014).
31. Lewis, E. A new standard food medium. 1960 Drosophila Information Service. in. *Cold Spring Harb Protoc.* **2014** (9), pdb.rec081414 (2014).
32. Bolker, B.M. *et al.* Generalized linear mixed models: a practical guide for ecology and evolution. *Trends Ecol Evol.* **24** (3), 127-135 (2009).
33. R Core Team *R: A Language and Environment for Statistical Computing.* at <https://www.R-project.org>. R Foundation for Statistical Computing. Vienna, Austria. (2015).
34. Schmittgen, T.D., Livak, K.J. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc.* **3** (6), 1101-1108 (2008).

35. Kalbfleisch, J.D., Prentice, R.L. *The Statistical Analysis of Failure Time Data, 2nd Edition.* Wiley-Blackwell. Hoboken, N.J. (2002).
36. Nuhanen, B. *Drosophila melanogaster, drawing.SVG.* at <https://commons.wikimedia.org/wiki/File:Drosophila-drawing.svg>. (2007).
37. Accountalive. *Example of a survival curve estimated by Kaplan-Meier method including 95% confidence limits.* at <https://commons.wikimedia.org/wiki/File:Kaplan-Meier-sample-plot.svg>. (2011).
38. Susi, H., Vale, P.F., Laine, A.-L. Host Genotype and Coinfection Modify the Relationship of within and between Host Transmission. *Am Nat.* **186** (2), 252-263 (2015).
39. Fellous, S., Duncan, A.B., Quillery, E., Vale, P.F., Kaltz, O. Genetic influence on disease spread following arrival of infected carriers. *Ecol Lett.* **15** (3), 186-192 (2012).